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Development of Innate Immunity During In Vitro Differentiation of Mouse Embryonic Stem Cells

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DEVELOPMENT OF INNATE IMMUNITY DURING IN VITRO DIFFERENTIATION OF MOUSE EMBRYONIC STEM CELLS

by

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ABSTRACT

DEVELOPMENT OF INNATE IMMUNITY DURING IN VITRO DIFFERENTIATION OF MOUSE EMBRYONIC STEM CELLS

by William Anthony D'Angelo

December 2017

Embryonic stem cells (ESCs) hold enormous promise for the goals of regenerative medicine and tissue engineering, and extraordinary progress has been made in defining conditions for differentiation to desired cell types. However, an often overlooked aspect of ESC biology is innate immunity, the ability of cells to detect and respond to pathogens and inflammatory cytokines. A number of recent studies by our lab and others have established that ESCs and other types of pluripotent cells from both mice and humans do not mount typical immune responses to viral or bacterial stimuli. There are also indications that various cell types differentiated from ESCs are also hyporesponsive, raising concerns for their suitability for therapeutic application. We have developed a model for the study of innate immunity during differentiation of mouse ESCs to fibroblasts (mESC-FBs). Using this model, the development of innate immune responses during in vitro differentiation was demonstrated by an increase in type I interferon (IFN) expression in response to viral stimuli, and an increased response to exogenous IFN, as compared with ESCs. The magnitude of responsiveness was further increased with continuous passaging, and this development could be accelerated by immune "priming" or exposure to low doses of immune stimulants during culturing. Differentiation correlated with a transition in functionality of the NFκB signaling pathway, a critical regulator of innate immune responses, from inactive in ESCs to a

functioning state in differentiated cells. In addition to antiviral responses, responsiveness to inflammatory cytokines was acquired during in vitro differentiation, which again relied on a functional NFκB pathway, as well as increased expression of cytokine receptors. Preliminary characterization of mESC-FBs revealed several similarities with mesenchymal stem cells (MSCs), including morphology and marker expression, differentiation capacity, and production of trophic and immunosuppressive mediators. Thus mESC-FBs are not only a valuable model to study the mechanisms of innate immunity development, but could serve as an unlimited source for therapeutically valuable MSC-like cells.

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I thank my labmates (and friends) Ruoxing Wang, Jundi Wang, Natalya Ortolano, Chandan Gurung, and Bohan Chen for their advice, help with experiments, and friendship, and for making the lab a great place to work every day. And I especially thank Dhiraj Acharya, for his valuable knowledge and assistance with viral infections.

DEDICATION

To my closest friends, whose support over the years has helped me more than they know. To my brothers, my grandparents, aunts, uncles, and cousins, who often had more faith in me than I did. And most of all to my parents, for their unwavering love and encouragement, and for setting an example of how to live a life to be proud of. I wouldn't be the person I am without them.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

LIST OF ABBREVIATIONS

PRR	pattern recognition receptor
PAMP	pathogen associated molecular pattern
TLR	toll-like receptor
dsRNA	double-stranded ribonucleic acid
LPS	lipopolysaccharide
NFκB	nuclear factor kappa light chain enhancer of activated B cells
IRF	interferon regulatory factor
IκB	inhibitor of κB
IKK	IκB kinase
IFN	interferon
ISG	interferon stimulated gene
ICAM-1	intercellular adhesion molecule-1
TNFα	tumor necrosis factor alpha
IL-6	interleukin-6
iNOS	inducible nitric oxide synthase
TGFβ	transforming growth factor-β
ESC	embryonic stem cell
hESC	human embryonic stem cell
PRR	pattern recognition receptor
mESC	mouse embryonic stem cell
MSC	mesenchymal stem cell
FB	fibroblast

RNAi	RNA interference
siRNA	small interfering RNA
EB	embryoid body
DMEM	Dulbecco's modified Eagle's medium
FBS	fetal bovine serum
LIF	leukemia inhibitory factor
mESC-DC	mouse embryonic stem cell differentiated cells
RA	retinoic acid
mESC-FB	mouse embryonic stem cell-derived fibroblasts
TB	toluidine blue
PBS	phosphate buffered saline
PKR	protein kinase R
MDA5	melanoma differentiation associated protein 5
LACV	La Crosse virus
CHIKV	chikungunya virus
RT-qPCR	reverse transcription quantitative polymerase chain reaction
SD	standard deviation
VEGF	vascular endothelial growth factor
bFGF	basic fibroblast growth factor
PDGF	platelet-derived growth factor
SCF	stem cell factor
HGF	hepatocyte growth factor
SDF-1	stromal cell-derived factor-1

COX2	cyclooxygenase 2
HO-1	heme oxygenase-1
CD105	cluster of differentiation 105

CHAPTER I – INTRODUCTION

Overview of Innate Immunity

Innate immunity is the collective term for the various systems organisms employ to detect and respond to pathogens in a non-specific manner, in contrast to adaptive immunity, which is pathogen-specific. The vertebrate innate immune response, as the first line of defense against infection, serves to limit the spread of the pathogen and to mobilize the adaptive immune system, which mounts a powerful and targeted response to future exposure to the same pathogen. Cells express an array of so-called pattern recognition receptors (PRRs), which detect pathogen-associated molecular patterns (PAMPs), broadly conserved motifs such as viral nucleic acids or bacterial flagellin. These PRRs activate downstream signaling pathways leading to the activation of a network of immune response genes that collectively fight the pathogen, alert neighboring cells of the danger, and recruit specialized immune cells to generate a tissue- or organism-wide response.

PRRs can be grouped into several families. The toll-like receptors (TLRs) are transmembrane proteins localized either at the cell surface or on endosomes that generally recognize bacterial membrane components or viral and bacterial nucleic acids (Yu et al., 2010). RIG-I-like receptors (RLRs) are cytosolic and are activated by viral double-stranded RNA (dsRNA) (Schlee, 2013). NOD-like receptors (NLRs) are also cytosolic, and are involved in sensing peptidoglycan components and formation of the inflammosome (Kanneganti et al., 2007). C-type lectin receptors (CLRs) are expressed mainly on immune cells and function in antigen presentation (Geijtenbeek and Gringhuis, 2009).

Several examples of PRRs are illustrated in Figure 1 below. TLR3 is expressed on the cell surface or in endosomes, and is activated by dsRNA, which is generated during the replication cycle of viruses. RIG-I also detects dsRNA, but is cytosolic rather than membrane bound. TLR4 detects lipopolysaccharide (LPS), a component of Gram-negative bacterial cell membranes. While different PRRs bind various different classes of ligands, and signaling downstream of the receptors is mediated by different proteins, PRR activation generally leads to activation of a set of transcription factors that can be considered master regulators of immune and inflammatory responses, namely the nuclear factor kappa-light-chain-enhancer of activated B-cells (NFκB) family, discussed below, and the interferon regulatory factor (IRF) family, part of the antiviral interferon system discussed later.

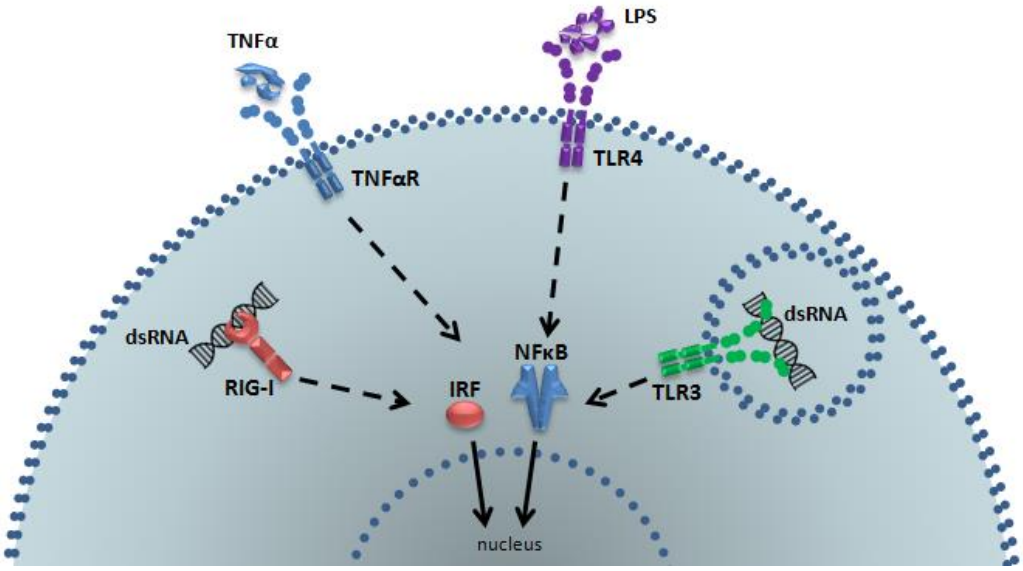

Figure 1. Overview of the receptors and transcription factors involved in innate immune and inflammatory responses.

Cells express an array of PRRs that detect broadly conserved molecular patterns found on various classes of pathogens. Activation of PRRs (e.g. RIG-I, TLR3, TLR4) and inflammatory cytokine receptors (e.g. TNFαR) by their cognate ligands (dsRNA, bacterial

endotoxin, and TNFα, respectively) induces signaling cascades that result in the activation and nuclear translocation of immune regulatory transcription factor families, including the IRF and NFκB families.

The NFκB family includes five members: p65/RelA, RelB, c-Rel, p50, and p52. Homo- or heterodimers of these proteins are sequestered in the cytosol by their interaction with inhibitor of κB (IκB) proteins. As illustrated in figure 2, upstream signaling induced by immune stimuli results in phosphorylation of the IκB kinase (IKK) complex, which in turn phosphorylates IκB, resulting in its ubiquitination and degradation and thereby exposing the nuclear localization sequence of NFκB dimers and allowing their translocation to the cytosol. NFκB then binds κB binding sites in target gene promoters to induce their transcription (Hayden et al., 2006). Of the many NFκB target genes so far identified, a large subset are antiviral and inflammatory cytokines, chemokines, cell adhesion molecules, and other genes involved in immune and inflammatory responses, illustrating the importance of this transcription factor for immune and stress responses in general.

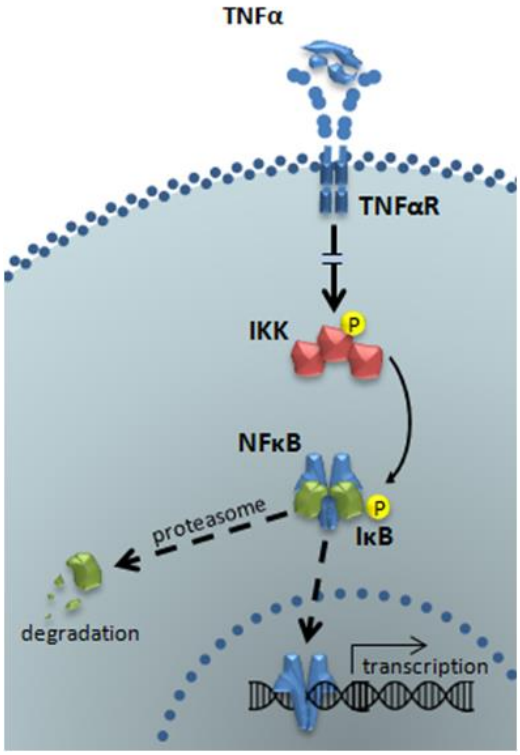

Figure 2. Mechanism of NFκB activation and nuclear translocation.

Signaling downstream of PRRs and inflammatory cytokine receptors results in the phosphorylation of IκB kinase (IKK), which in turn phosphorylates the inhibitor of kappa B (IκB) protein. Phosphorylated IκB is then ubiquitinated and degraded, freeing NFκB to translocate to the nucleus and activate the transcription of hundreds of genes that govern immune and inflammatory responses, such as cytokines, chemokines, genes that regulate adhesion, and cell cycle regulators.

Antiviral Innate Immune Response

The hallmark of the antiviral innate immune response is the production of a class of cytokines called interferons (IFN). IFNs can be grouped into three classes: type I, II, and III. Type I IFNs (IFNα/β/ε/κ/τ/ω, of which IFNα and IFNβ are the best characterized) can be expressed by and act on most cell types to illicit antiviral responses (González-Navajas et al., 2012). IFNγ is the sole member of the type II class. Its expression is confined to certain immune cells (T-lymphocytes, NK cells), and it is involved in multiple processes such as antigen presentation, regulation of adaptive immune cell

behavior, and apoptosis(Schroder et al., 2004). Type III IFNs are not well-studied and are structurally distinct from the other two classes, but appear to mediate similar effects as type I IFNs(Randall and Goodbourn, 2008). Although the exact pathways to induction of IFNs are stimulus- and cell type-dependent, generally the detection of viral or bacterial components by PRRs leads to activation of members of the IRF family of transcription factors which, along with NFκB, translocate to the nucleus and bind to type I IFN promoters to induce their expression(González-Navajas et al., 2012).

Once expressed, IFNs are secreted into the extracellular space and act in autocrine and paracrine manner by binding to cell surface IFN receptors. This results in activation of the JAK-STAT signaling pathway, leading to nuclear translocation of STAT dimers, which along with IRF9 form the interferon stimulated gene factor 3 (ISGF3) complex. This complex then induces the transcription of hundreds of interferon-stimulated genes (ISGs) to induce the so-called antiviral state (Randall and Goodbourn, 2008). Some examples of well-characterized ISGs include protein kinase R (PKR), a sensor of double-stranded RNA whose activation results in a halt on protein synthesis; 2'-5'-oligoadenylate synthetase 1 (OAS1), which activates RNase L, an enzyme that degrades both cellular and viral RNA; and Mx, which binds to viral structural proteins to restrict assembly and replication (Randall and Goodbourn, 2008). Collectively, the IFN-mediated antiviral response acts through multiple mechanisms to inhibit viral replication, promote apoptosis of infected cells, and activate the adaptive immune system.

Inflammation

Acute inflammation is a coordinated tissue response to infection or injury characterized by the recruitment from the circulation of leukocytes and blood

components, resulting in the five cardinal signs: redness, pain, heat, swelling, and loss of function (Ryan and Majno, 1977). This brief review will focus on the inflammatory response in the context of infection, although it should be noted that inflammation is a key component of many other non-infectious pathologies such as cancer and auto-immune diseases, and may be more accurately considered as a reaction to any disturbance of tissue homeostasis, no matter the cause (Medzhitov, 2010).

Tissue-resident sentinel cells (i.e. macrophages, mast cells, and stromal cells such as fibroblasts) are generally the first to encounter infectious agents. At the cellular level, detection of a pathogen by PRRs results in the expression of a network of immune response genes by the previously discussed mechanisms. Several of these response genes are so-called inflammatory cytokines, paracrine signaling molecules which themselves activate further subsets of response genes to coordinate an intricate set of behaviors by the various cell types of the tissue. For example, products of inflammatory signaling include vasoactive substances such as histamine that affect vascular tone and increase the permeability of blood vessels; adhesion molecules such as ICAM-1 that allow leukocyte attachment to blood vessel walls to promote tissue infiltration; various chemokines that attract circulating immune cells to the site; and proteases that degrade the extracellular matrix to allow infiltration of these immune cells (Nathan, 2002). When leukocytes (mainly neutrophils in the acute phase) reach the site of infection, they release large amounts of reactive oxygen species and reactive nitrogen species to target and destroy the invading pathogen (Medzhitov, 2008). As all of these processes also cause perturbation of the normal tissue environment and collateral damage to host cells, precise control of the inflammatory response is crucial. Virtually every level of the process is subject to

myriad regulatory mechanisms and feedback loops, such that the response is dynamic and tailored to the nature and severity of the initiating stimulus (Nathan, 2002).

Tissue-resident macrophages play a major role in all phases of inflammation. Detection of a pathogen by macrophage PRRs or activation by inflammatory cytokines released from other infected tissue cells results in polarization of macrophages toward a pro-inflammatory M1 phenotype, characterized by high production of TNFα, IL-6, IL-1, chemokines, prostaglandins, and iNOS. After the pathogen is cleared, macrophages adopt an anti-inflammatory M2 phenotype to promote the resolution phase of the acute inflammatory response. M2 polarized macrophages produce IL-10, TGF-β, and arginase-1, and facilitate tissue regeneration, the clearance of neutrophils from the tissue, and the restoration of tissue homeostasis (Chung and Son, 2014).

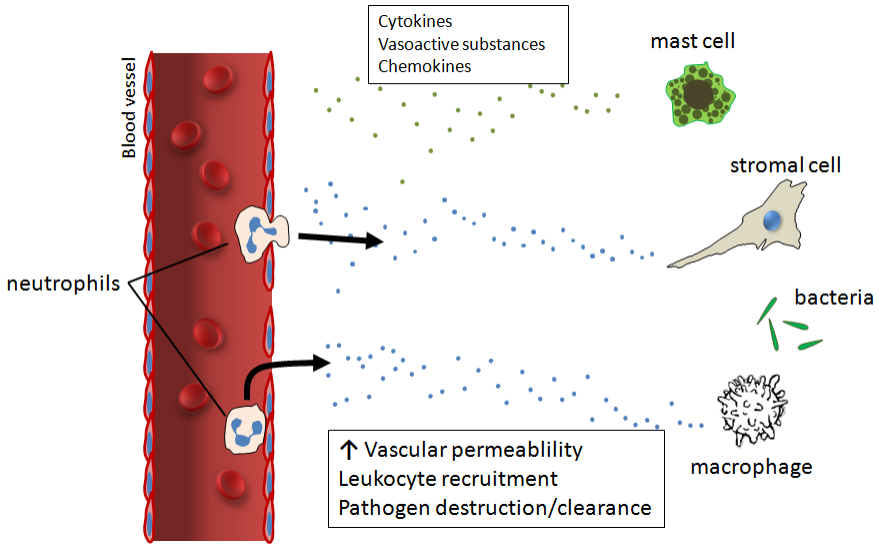

Figure 3. Overview of acute inflammatory response to pathogen detection.

Pathogens are detected through PRRs expressed by stromal cells such as fibroblasts or tissue-resident immune cells such as mast cells or macrophages, resulting in the production of inflammatory cytokines and chemokines, as well as vasoactive mediators. This leads to activation of capillary endothelial cells, increased vessel permeability, and attachment and tissue infiltration of circulating immune cells (e.g. neutrophils) to the site of infection. Once the pathogen is cleared, production of anti-inflammatory cytokines, growth factors, and other mediators promotes wound healing and a return to homeostasis.

Embryonic Stem Cells and Innate Immunity

Embryonic stem cells (ESCs) are derived from inner mass cells of an early developmental stage embryo called a blastocyst (Figure 4). The blastocyst is composed of an outer layer of trophoblasts, fibroblast-like cells which eventually form the placenta, and inner mass cells which give rise to the embryo proper. Inner mass cells can be isolated from the blastocyst and cultured in vitro, where they are termed ESCs. These cells have two defining characteristics: pluripotency, or the ability to differentiate into all cell lineages from each of the three germ lines (endoderm, mesoderm, ectoderm); and self-renewal, or the capacity to proliferate indefinitely without losing pluripotency, given the proper culture conditions(Brook and Gardner, 1997). These characteristics raise the possibility of regenerative medicine and cell replacement therapy, and this potential has been the main driving force behind ESC research(Chen and Daley, 2008).

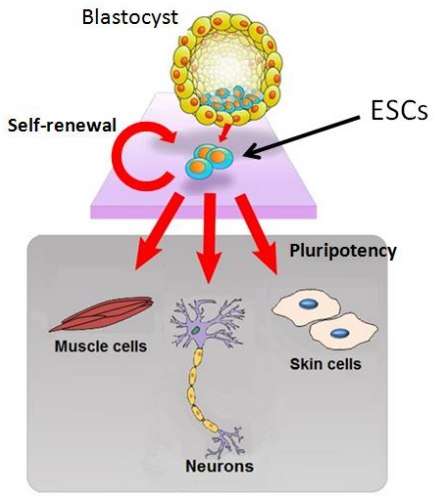

Figure 4. Derivation and differentiation of ESCs.

The blastocyst stage of early embryonic development consists of an outer layer of trophoblast cells, which form the placenta and other extra-embryonic tissues, and inner mass cells, the precursors for all future cells of the embryo. Inner mass cells can be isolated and propagated in vitro, where they are termed ESCs, and are characterized by the ability to differentiate to any cell type from all three germ layers (pluripotency), and the capacity for unlimited proliferation without differentiation (self-renewal).

The stem cell state is maintained by a complex network of transcription factors collectively called pluripotency factors, including Oct4, Sox2, Nanog, and other proteins as well as regulatory microRNAs(Chen and Daley, 2008). This network serves to maintain its own expression and inhibit transcription of genes that promote differentiation. Mechanistically, pluripotency factors regulate transcription of histone modifying enzymes (Boyer et al., 2005) and interact directly with chromatin remodeling proteins(Wang et al., 2006) in order to create a unique epigenetic landscape that maintains pluripotency but is also primed for differentiation once the proper cues are given(Chen and Daley, 2008).

Although the first reports of hyporesponsiveness to immune stimuli by pluripotent cells appeared decades ago (Burke et al., 1978; Swartzendruber and Lehman, 1975; Swartzendruber et al., 1977), only recently has the subject begun to attract more attention. While they are susceptible to viral and bacterial infection-induced cytotoxicity, infected ESCs do not respond with expression of interferons and inflammatory cytokines as do differentiated cells. hESCs did not respond to challenge with live bacteria or with various TLR ligands (Chen et al., 2010; Földes et al., 2010), and mESCs were also found to lack immune gene induction when challenged with viral (Wang et al., 2013; Wash et al., 2012) or bacterial stimuli (Yu et al., 2009). Similar results have been reported in induced pluripotent stem cells (iPSCs) (Chen et al., 2012) and embryonal carcinoma cells (Harada et al., 1990), indicating that the lack of innate immune response to pathogenic stimuli is general to pluripotent cells. A major unresolved question is whether this deficiency is a consequence of the unique mechanisms that maintain pluripotency and

self-renewal, or is instead a protective mechanism to prevent catastrophic damage to the developing embryo resulting from a robust immune response (Guo et al., 2015).

Aside from the ability to recognize and respond to viral or bacterial pathogens, the innate immune system can also discriminate between self and non-self cells. The ability of a cell to be recognized as foreign is referred to as immunogenicity. Self versus non-self recognition relies largely on similarity of major histocompatibility complex (MHC) proteins found on the surface of all cells. Studies on the immunogenicity of ESCs and their derived cells have reported conflicting results—some researchers found that hESCs and mESCs did not express MHC and thus escaped immune detection (Drukker et al., 2002; Magliocca et al., 2006), and did not activate T-cell proliferation, either before or after differentiation (Li et al., 2004). But other studies (Boyd and Wood, 2009; Ma et al., 2010; Swijnenburg et al., 2005) found that immunogenicity increases with differentiation and under inflammatory conditions, indicating that more study is needed to gauge the immunogenic potential of pluripotent cells and their derivatives.

Mesenchymal Stem Cells and Innate Immunity

Mesenchymal stem cells (MSCs) are a class of so-called "adult stem cells", meaning that they have the ability to differentiate to multiple cell types and the capacity for self-renewal, but unlike ESCs they persist in the organism past the embryonic stage. MSCs were originally characterized from bone marrow (Friedenstein et al., 1976), but similar cells have now been isolated from a wide variety of tissues, including adipose tissue (Hedrick et al., 2002), dental pulp (Gronthos et al., 2000), and umbilical cord blood (Majore et al., 2009), among others. MSCs are characterized by their ability to differentiate to osteocytes, chondrocytes, and adipocytes (bone, cartilage, and fat cells,

respectively), although some studies have reported neurogenic (Qian and Saltzman, 2004), endothelial (Liu et al., 2007), and myogenic (Wakitani et al., 1995) differentiation as well. In contrast to ESCs, which are pluripotent, the differentiation capacity of MSCs is restricted to a small number of cell types; thus they are termed multipotent. While this might limit the range of potential applications for MSCs in regenerative medicine, it also makes them safer for transplantation than ESCs, as MSCs are non-tumorigenic. Indeed, MSCs have already been evaluated in a number of clinical trials for the treatment of bone and connective tissue injuries (U.S. National Institutes of Health, 2016).

In addition to their differentiation capacity, MSCs have been shown to have potent immunosuppressive and anti-inflammatory properties. MSCs can suppress inflammation by inhibiting or reversing the activation of immune cells (e.g. macrophages, dendritic cells, T-lymphocytes, mast cells (Kim et al., 2015; Kimbrel et al., 2014; Di Nicola et al., 2002; Ren et al., 2008)), accomplished both through cell contact-dependent mechanisms and the production of a variety of paracrine mediators, including nitric oxide, prostaglandin E2, and cytokines such as TGF-β (Bernardo and Fibbe, 2013). These mechanisms are engaged or upregulated when the cells are exposed to innate immune or inflammatory stimuli, especially IFNγ (Ren et al., 2008). It is this anti-inflammatory property that has made MSCs most attractive for potential clinical usage, with hundreds of clinical trials currently ongoing (U.S. National Institutes of Health, 2016), many for the treatment of inflammatory diseases such as graft-versus-host disease, ulcerative colitis, and type I diabetes.

Role of Fibroblasts in Innate Immune Responses

Fibroblasts (FBs) are cells of mesodermal origin whose characteristic function is the deposition of extracellular matrix to give structural support to tissues. When cultured in vitro they are plastic-adherent and typically have a spindle-shaped, flattened morphology. Although FBs were first described by Virchow over a century ago (Virchow, 1858), their important role in tissue homeostasis and immunity is still relatively under-appreciated. As a major cellular component of many tissues, FBs may serve as "sentinel cells", important for detecting pathogens and initiating immune responses by production of cytokines and chemokines (Smith et al., 1997), and can act as antigen presenting cells (APCs) (Kündig et al., 1995). FBs are also involved in the resolution of immune responses, exerting immunosuppressive effects that promote tissue clearance by immune cells and wound healing and revascularization (Jordana et al., 1994; Van Linthout et al., 2014; Smith et al., 1997).

It is interesting to note that studies directly comparing MSCs with FBs found similar suppressive effects on immune cells, mediated through similar mechanisms (Haniffa et al., 2007; Wada et al., 2011). FBs have also been reported to differentiate to bone, cartilage, and fat (Covas et al., 2008; Lorenz et al., 2008), and display morphology and marker expression similar to MSCs (Wagner et al., 2005). Although classically isolated from bone marrow, cells with characteristics of MSCs have been isolated from a wide variety of tissue sources, and are now thought to occupy a perivascular niche in the microvasculature of most tissues (Crisan et al., 2008). This fact, taken together with their other similarities, has led some to argue that the two are related cell types, or that the MSC phenotype is simply a functional state adoptable under certain conditions by

FBs/stromal cells from virtually all tissues (Caplan, 2008; Haniffa et al., 2009; Hematti, 2012; Ulrich, 2012).

CHAPTER II – OBJECTIVE AND SIGNIFICANCE

One of the major goals of stem cell research is regenerative medicine, or the therapeutic use of stem cells or their differentiated cells to repair or replace damaged or dysfunctional cells, tissues, or organs. While great progress has been made in defining the conditions for in vitro differentiation of ESCs to various specialized cell types, the characterization of these ESC-differentiated cells usually does not take into account their immunocompetency. This is an important concern, but is often overlooked since in vitro cell culture is carried out under sterile conditions. Although innate immunity has been assumed to function in all cell types, research from our lab and others has shown that ESCs exhibit little or no response to a wide variety of immune and inflammatory stimuli, including live viruses (Wang et al., 2013) and bacteria (Földes et al., 2010), viral or bacterial products such as dsRNA or LPS, or inflammatory cytokines such as TNFα (Kim et al., 2008). From these data it is apparent that the immune/inflammatory response is developmentally regulated. This finding has important implications for the use of stem cells or their derived cells in regenerative medicine, as transplantation of cells that are not fully immunocompetent could increase susceptibility to infection. Conversely, cells that are unable to contribute to a pro-inflammatory response could be beneficial during transplantation into an already inflamed surgical wound. Thus, my objective is to fully characterize the immunoproperties of ESC-derived differentiated cells, which will not only provide basic insight into the development of innate immunity, but also is essential information for evaluating the functionality of these cells for clinical use.

Compared with ESCs, MSCs have progressed much farther toward clinical implementation. Because ESCs are pluripotent, they carry a risk of tumor formation after

transplantation, and must be fully differentiated in vitro to a desirable cell type before use. MSCs on the other hand do not form tumors, and their immunosuppressive and trophic characteristics, in addition to their differentiation capacity, make them applicable to a wide range of diseases without the need for extensive in vitro manipulation. However, their harvest from patients requires invasive procedures; donor variability could lead to significant differences in clinical efficacy; and their relative scarcity in the tissue necessitates ex vivo expansion to obtain a clinically useful number of cells. I have demonstrated in the following studies that ESCs could serve as an unlimited and highly consistent source from which to derive cells with the beneficial characteristics of MSCs, which would represent a significant achievement in regenerative medicine.

CHAPTER III - DEVELOPMENT OF ANTIVIRAL INNATE IMMUNITY DURING IN VITRO DIFFERENTIATION OF MOUSE EMBRYONIC STEM CELLS

Introduction

Embryonic stem cells (ESCs) are characterized by their potential to differentiate into different cell lineages and their unlimited capacity to self-renew. These properties make them a promising cell source for regenerative medicine (Wobus and Boheler, 2005). Intensive studies have led to the generation of various cell types from ESCs (Keller, 2005). However, ESC-derived cells are usually characterized by their morphology, marker expression, and cell type-specific functions. In most cases, it is not clear whether in vitro differentiated cells are functionally equivalent to their in vivo counterparts. Several recent studies reported that ESC-derived endothelial cells, cardiomyocytes, and smooth muscle cells from both human and mouse ESCs (hESCs and mESCs) show limited or no response to a wide range of infectious agents and inflammatory cytokines (Földes et al., 2010; Glaser et al., 2011; Rajan et al., 2008; Zampetaki et al., 2006). This is in contrast to their naturally-differentiated counterparts, which are exquisitely sensitive to infectious and inflammatory stimuli. These findings raise important questions for the therapeutic application of ESC-derived cells since they would likely be exposed to pathogens and inflammatory cytokines when used in tissue implantation.

The immune system of vertebrates consists of innate and adaptive immunity. Innate immunity responds to a broad range of pathogens in a nonspecific manner and provides the first line of defense through several mechanisms, including inflammation and innate immune response, whereas the adaptive immunity provides defense in a

pathogen-specific manner through highly specialized immune cells. The innate immunity is well-developed in most, if not all somatic cells (Sen, 2001). However, recent studies demonstrated that hESCs do not respond to a wide range of infectious agents (Chen et al., 2010; Földes et al., 2010). Similarly, mESCs are susceptible to the cytopathic effect of bacterial and viral infection, but they do not show immune responses typically seen in differentiated cells (Wash et al., 2012; Yu et al., 2009). Our lab recently reported that mESCs are unable to express type I interferon (IFN) (Wang et al., 2013, 2014a) and have attenuated responses to these cytokines (Wang et al., 2014b). Therefore, the IFN system, which is the central part of innate immunity in differentiated somatic cells (Samuel, 2001), is not fully functional in ESCs. Together with the similar findings in induced pluripotent stem cells (Chen et al., 2012) and embryonal carcinoma (Harada et al., 1990), an underdeveloped antiviral innate immunity represents an intrinsic property of all pluripotent cells (reviewed in (Guo et al., 2015)).

While the physiological implications of the underdeveloped innate immunity in ESCs are not yet completely understood, we can speculate from different perspectives. ESCs normally reside in the womb where they have limited exposure to pathogens and are likely protected by the mother's immune system (Levy, 2007). However, a different conjecture could be made based on the fact that immune and inflammatory responses often have multiple impacts, including various adverse effects on infected cells, such as cell cycle inhibition or cell death (García et al., 2007; Samuel, 2001). These negative effects on ESCs could be detrimental to the organism's development since they are the progenitors for all ensuing tissues. On the other hand, it would be equally disastrous if ESCs do not have an effective antiviral mechanism to prevent viral infection. The recent

discovery of RNA interference (RNAi) in mESCs offers a plausible solution to this dilemma (Maillard et al., 2013). RNAi is a major antiviral mechanism in plants and invertebrates, which lack IFNβ-based innate antiviral immunity. It has been uncertain whether RNAi functions in mammals, where a well-developed IFN system can mount multiple forms of antiviral responses (Cullen et al., 2013; Pare and Sullivan, 2014). Using mouse models, it has been recently demonstrated that the RNAi mechanism is functional in mESCs, but its efficiency is significantly diminished in differentiated cells (Li et al., 2013; Maillard et al., 2013). An emerging paradigm is that mammals may have adapted different antiviral strategies at different stages of development. By utilizing virus-specific and short-lived siRNA derived from invading viruses, a developing organism may prevent viral infection in ESCs and avoid potential negative effects associated with IFN, while later-developed IFNβ-based antiviral mechanisms in somatic cells may confer powerful antiviral activities at multiple levels (reviewed in (Pare and Sullivan, 2014)).

Based on the lack of effective responses of ESCs to various pathogens, it is apparent that the innate immunity is not ‘‘innate’’ to ESCs and it must be ‘‘acquired’’ by somatic cells during the process of organism development. It is conceivable that the attenuated immune and inflammatory responses in in vitro ESC-derived cells is likely attributable to the lack of the same function in ESCs and the process of their differentiation. This not only raises concerns for their therapeutic application, but also brings up several questions that overlap the basic sciences of ESC biology, developmental biology, and immunology. In this study, I attempted to address the question of whether the commonly used in vitro differentiation methods can generate ESC-derived cells with active antiviral innate immunity. These findings provide valuable

insights into this question and could be instructive for designing strategies that can generate ESC-derived cells with desired level of innate immunity for their use in regenerative medicine.

Methods

Cell culture

D3 and DBA252 mESCs were cultured in DMEM with 15% FBS, 20 ng/mL LIF, 0.2 mM L-glutamine, 0.1 mM 2-mercaptoethanol, 0.1 mM MEM nonessential amino acids, 100 U/mL penicillin, and 100 mg/mL streptomycin. C3H10T1/2 cells [10T1/2, a line of mouse embryonic fibroblasts (FBs), ATCC] were cultured in DMEM with 10% FBS and 100U/mL penicillin and 100 mg/mL streptomycin. All cells were maintained at 37°C in a humidified incubator with 5% CO_2.

mESC differentiation

Two common methods for in vitro differentiation of mESCs were used in this study. The first was ESC differentiation through embryoid body (EB) formation as previously described (Guo et al., 2007). Briefly, mESCs (1×10^5 cells/mL) were suspended in a bacterial culture dish where they clumped and formed EBs. After incubation for 24 h, the medium was changed to leukemia inhibitory factor (LIF)-free medium containing 15% FBS to initiate differentiation. After incubation for 5 days, EBs were transferred to gelatin-coated cell culture dishes where the cells within the EBs grew out to form a monolayer. The cells derived from the monolayer, designated as mESC-differentiated cells (mESC-DCs), represent a mixed population of multiple cell types as previously characterized (Guo et al., 2007).

In the second method, mESC differentiation was induced by retinoic acid (RA) according to the published method with some modifications (Wang et al., 2014b). Briefly, mESCs grown in low density (30%–40% confluence) in a cell culture dish were treated with 1 mM RA and the medium was refreshed three times during a 10-day period of differentiation. The differentiated cells formed a compact monolayer, which was trypsinized and disaggregated. The single cell suspension was reseeded in an uncoated cell culture dish, to which FBs quickly attached within ~30 min. The floating cells in the medium were removed by changing the medium. This process was repeated two to three times and the resulting cells showed the typical morphology of FBs (designated as passage one, p1) and expressed several cell markers common to FBs as previously characterized (Wang et al., 2014b). Thus, mESC-differentiated FBs were named as mESC-FBs (i.e. D3-FBs and DBA-FBs, differentiated from D3 and DBA mESCs, respectively). These cells were further propagated in DMEM with 10% FBS to different passages. The cells from a specified passage or from several consecutive passages were used for related experiments as described in individual experiments.

Cell proliferation, viability, and cell cycle analysis

Cell proliferation and viability were determined by toluidine blue staining. Briefly, cells were fixed with methanol for 10 minutes at RT, then air dried. Cells were stained with 1% TB in dH2O for 30 minutes, rinsed with tap water, and the stain was extracted with 2% SDS. The absorbance at 630 nm of stained cells was measured with a BioTek ELx800 Microtiter Plate Reader. The absorbance values, which correlate with the amount of cellular content (proteins/DNA), were used as an indirect measurement of cell number.

For cell cycle analysis, cells were trypsinized to a single cell suspension, then fixed with 80% cold ethanol for 15 minutes at 4°C. Cells were washed with PBS and flow cytometry was performed after the cells were stained with propidium iodide. The cell cycle profiles were generated with the CFlow software as previously described (Guo et al., 2010).

Cell treatment

The 10T1/2 cells and mESC-FBs were seeded at 70%–80% confluence or otherwise specified. The antiviral responses induced by polyinosinic:polycytidylic acid (polyIC, a synthetic dsRNA used as a viral analog) were performed by transfection of the cells with polyIC (300 ng/mL or otherwise specified) using DharmaFECT reagent (Thermo Scientific). The cellular responses to type I IFN were determined with recombinant IFNα (IFNα-2, 1 x10^8 U/mg; eBioscience) and recombinant IFNβ (5 x 10^8 U/mg; PeproTech) as previously described (Wang et al., 2014b). To inhibit the expression or activation of dsRNA-dependent protein kinase (PKR), the cells were transfected with siRNA targeting PKR (Santa Cruz Biotechnology) or treated with a PKR inhibitor (imidazolo-oxindole, C16; Sigma) at the concentration of 1 mM, as previously described (Wang et al., 2013).

Viral stock preparation and cell infection

La Crosse virus (LACV, SM6 v3) and chikungunya virus (CHIKV, LR 2006 OPY1 strain) were propagated in Vero cells (African green monkey kidney cell line, ATCC). Titers of virus stocks were determined in Vero cells by plaque assay as previously described (Bai et al., 2005). The cells were infected with LACV and CHIKV

at multiplicity of infection of 5 and 1, respectively, or otherwise specified in individual experiments.

Real-time quantitative polymerase chain reaction

Total RNA was extracted using TRI Reagent (Sigma). cDNA was prepared using Moloney Murine Leukemia Virus Reverse Transcriptase (Sigma). Real-time quantitative polymerase chain reaction (RT-qPCR) was performed using SYBR Green Ready Mix (Bio-Rad) on a MX3000P RT-PCR system (Stratagene), as previously described (Guo et al., 2007). The mRNA levels from RT-qPCR were calculated using the comparative Ct method (Pfaffl, 2001). β-actin was used as a calibrator for the calculation of relative mRNA of the tested genes. In the experiments with viruses, 18S rRNA was used as a calibrator due to the degradation of β-actin caused by viral infection. The sequences of the primer sets are listed in Table 1.

Table 1

RT-qPCR primer sequences for mouse genes

Gene	Sequence (forward)	Sequence (reverse)
β-actin	CATGTACGTAGCCATCCAGGC	CTCTTTGATGTCACGCACGAT
18s rRNA	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
RIG-I	ATTCAGGAAGAGCCAGAGTGTC	GTCTTCAATGATGTGCTGCAC
TLR3	CTTGCGTTGCGAAGTGAAGAA	CCAATTGTCTGGAAACACCCC
MDA5	CGATCCGAATGATTGATGCA	AGTTGGTCATTGCAACTGCT
CD14	CTCTGTCCTTAAAGCGGCTTAC	GTTGCGGAGGTTCAAGATGTT
TLR4	TGCACTGAGCTTTAGTGGTTGC	GACCCATGAAATTGGCACTCAT
TNFR1	CCGGGAGAAGAGGGATAGCTT	TCGGACAGTCACTCACCAAGT
ICAM1	GGCATTGTTCTCTAATGTCTCCG	GCTCCAGGTATATCCGAGCTTC
IL-6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
MCP-5	ATTTCCACACTTCTATGCCTCCT	ATCCAGTATGGTCCTGAAGATCA
iNOS	CAGCACAGGAAATGTTTCAGC	TAGCCAGCGTACCGGATGA
TNFα	CAGGCGGTGCCTATGTCTC	CGATCACCCCGAAGTTCAGTAG
IL-10	CTCTGACCGCTGTGTACGAT	CAATGGTGGTCTGCTGGTTC
p65 NFκB	AGGCTTCTGGGCCTTATGTG	TGCTTCTCTCGCCAGGAATAC

Table 1 (Continued)

IFNβ	**CCCTATGGAGATGACGGAGA**	**ACCCAGTGCTGGAGAAATTG**
MyD88	TCCGGCAACTAGAACAGACAGACT	GCGGCGACACCTTTTCTCAAT
MD-2	CGCTGCTTTCTCCCATATTGA	CCTCAGTCTTATGCAGGGTTCA
COX2	TGAGCAACTATTCCAAACCAGC	GCACGTAGTCTTCGATCACTATC
HO-1	CACAGCACTATGTAAAGCGTC	TGTGCAATCTTCTTCAGGACC
TGFβ	ATCCTGTCCAAACTAAGGCTCG	ACCTCTTTAGCATAGTAGTCCGC
PDGFB	CATCCGCTCCTTTGATGATCTT	GTGCTCGGGTCATGTTCAAGT
HGF	ATGTGGGGGACCAAACTTCTG	GGATGGCGACATGAAGCAG
SDF-1	TGCATCAGTGACGGTAAACCA	CACAGTTTGGAGTGTTGAGGAT
SCF	CCCTGAAGACTCGGGCCTA	CAATTACAAGCGAAATGAGAGCC
CTGF	GGGCCTCTTCTGCGATTTC	ATCCAGGCAAGTGCATTGGTA
VEGFA	GGAGATCCTTCGAGGAGCACT	GGCGATTTAGCAGCAGATATAA
bFGF	GCGACCCACACGTCAAACTA	CCGTCCATCTTCCTTCATAGC
OCN	CTGACCTCACAGATGCCAAG	GTAGCGCCGGAGTCTGTT
SOX9	AGTACCCGCATCTGCACAAC	ACGAAGGGTCTCTTCTCGCT
C/EBPα	CAAGAACAGCAACGAGTACCG	GTCACTGGTCAACTCCAGCAC

Flow cytometry

Cells were fixed with 4% paraformaldehyde for 15 minutes at 4°C, washed three times with PBS, and permeabilized with 0.05% Tween 20 in PBS for 10 minutes at RT. Cells were then washed with PBS, blocked in 2% BSA in PBS for 30 minutes at RT and incubated overnight at 4°C with antibodies against: TLR3 (toll-like receptor 3, BioLegend), IFNβ (BioLegend), RIG-I (retinoic acid–inducible gene I, Santa Cruz Biotechnology), PKR (Santa Cruz Biotechnology). With the exception of anti-TLR3 antibodies, which were preconjugated with PE (phycoerythrin), all other antibodies were detected with secondary antibodies that were either conjugated with FITC (fluorescein isothiocyanate) or rhodamine. The cells were then analyzed with an Accuri C6 flow cytometer (BD Biosciences). The fluorescence intensity, which correlates with the protein level detected with its specific antibody, was determined with the CFlow software (Wang et al., 2013).

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde for 15 minutes at 4°C, then washed three times with PBS and permeabilized with 0.1% Triton-X 100 in PBS for 10 minutes at RT. Cells were again washed three times with PBS, then blocked for 30 minutes in 2% BSA in PBS and stained with primary antibodies overnight at 4°C. Cell were then washed with 2% HS in PBS and stained with secondary antibodies for 1 h at RT. The cellular location of nuclear factor kappa B (NFκB) was determined with a FITC-conjugated RelA antibody (Santa Cruz Biotechnology). The cells infected with CHIKV were detected with a monoclonal anti-CHIKV antibody 3585 (Abcam). The cells infected with LACV were detected with monoclonal antibodies against the Gc protein encoded by LACV genome (a gift from Dr. Samantha Soldan, the University of Pennsylvania School of Medicine) (Soldan et al., 2010). The cells were examined under an LSM 510 laser-scanning confocal microscope (Zeiss).

Statistical analysis

For statistical analysis, data are presented as the mean ± SD derived either from three independent experiments or from a representative experiment performed in triplicate that was performed at least twice with similar results. Statistical analysis was performed using a two-tailed and paired student's t-test. Differences are considered statistically significant when $P < 0.05$.

Results

mESC-DCs through EB formation have a limited capacity to express IFN

The stem cell state of mESCs is maintained by LIF. The basic principle of in vitro differentiation is that LIF withdrawal from the culture medium triggers spontaneous

differentiation while the cell fate and differentiation rate can be influenced by different agents and growth conditions (Keller, 2005). Undifferentiated mESCs grow in colonies in a cell culture dish. When deprived of cell adhesion and cultured in suspension, mESCs grow in aggregates and form structures known as EBs since they resemble an early embryo in structure and differentiation process (Guo et al., 2007). As illustrated in Figure 5A, mESCs were allowed to differentiate in the form of 5-day-old EBs (5dEBs) followed by further differentiation in a monolayer formed from the outgrowth of EBs (5dEB-5dMo). When differentiated cells in the monolayer were trypsinized and replated into a new culture dish, they attached and formed a new cell monolayer consisting of different cell types (collectively designated as mESC-DCs, Figure 5A). Since mESCs are unable to express IFNβ in response to synthetic viral RNA analogs and live viral infection (Wang et al., 2013, 2014a), we examined whether differentiation would change this deficiency. As shown in Figure 5B, both polyIC (a synthetic dsRNA that has been commonly used as a viral RNA mimic) and LACV induced robust IFNβ expression in 10T1/2 cells (naturally-differentiated FBs) as a positive control (>3,000-fold activation), whereas mESCs were basically unresponsive. Although mESC-DCs showed a notable increase of IFNβ expression compared to mESCs in response to polyIC (~500-fold activation) or LACV infection (~40-fold activation), such induction is substantially lower than 10T1/2 cells.

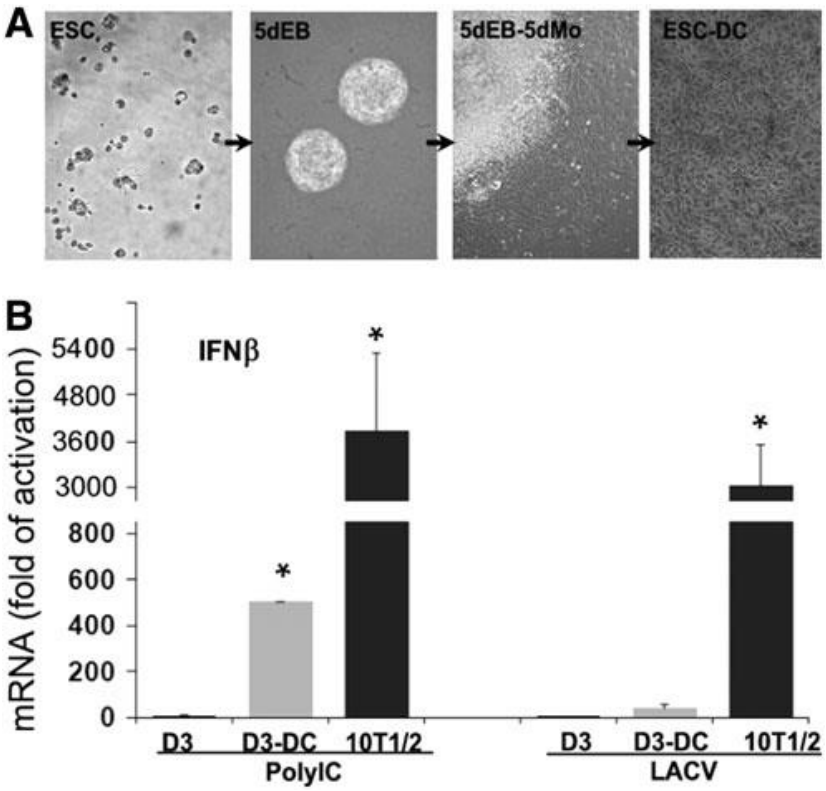

Figure 5. mESC-DCs generated from EB formation express limited IFNβ in response to polyIC and viral infection.

(A) Representative process of mESC differentiation through EB formation. The cells were photographed with a phase contrast objective lens (10x). (B) PolyIC transfection- and LACV infection-induced expression of IFNβ in D3, D3- DCs, and 10T1/2 cells. The mRNA levels of IFNβ were determined by RT-qPCR 24 h posttreatment. The results are expressed as fold activation, where the mRNA level in the control of each set of experiments is designated as 1 (not shown). *P< 0.05, compared with D3 cells.

Characterization of mESC-FBs generated through RA induction

mESC-DCs generated from EB differentiation contain mixed cell types (Guo et al., 2007). Therefore, the limited IFNβ expression shown in mESC-DCs (Figure 5B) may reflect a common property of different types of cells in the preparation. The 10T1/2 cells are FBs isolated from a 14–17 day old C3H mouse embryo (Figure 6A, C3H-ME) (Pinney and Emerson, 1989; Reznikoff et al., 1973). For a direct comparison, mESC-FBs were generated from two independent mESC cell lines (D3 and DBA) through RA-induced differentiation. RA is a vitamin A derivative that regulates several developmental

processes during embryogenesis and strongly induces ESC differentiation in vitro (Keller, 2005). As shown in Figure 6A, mESC-FBs (D3-FBs and DBA-FBs) completely lost the morphology of ESCs and formed a uniform monolayer that is very similar to that formed by 10T1/2 cells. mESCs are characterized by their high proliferation rate with about 60% of cells in the S phase, whereas the proliferation of mESC-FBs was dramatically slowed down with an overall similar cell cycle profile to 10T1/2 cells as indicated by the reduced cell population at S and G2/M phases (Figure 6B), although D3-FBs and DBA-FBs had slightly higher growth rate than 10T1/2 cells (Figure 6C). More importantly, mESC-FBs expressed several FB markers with similar patterns to 10T1/2 cells as previously described (Wang et al., 2014b). mESC-FBs can be continuously cultured for at least 15 passages. Cryopreserved cells can be further subcultured up to 55 passages. mESC-FBs at different passages exhibit consistent morphology when regularly split every 3–4 days. More than 95% of the cells express smooth muscle α-actin as a marker of FBs, similar to 10T1/2 cells (Wang et al., 2014b). Since D3-FBs and DBA-FBs are very similar in the properties that have been examined, the experiments to determine their antiviral mechanisms were mainly performed with D3-FBs (the parental D3 ESCs are one of the commonly used cell lines in the literature), whereas DBA-FBs were used for confirmative analysis in select experiments.

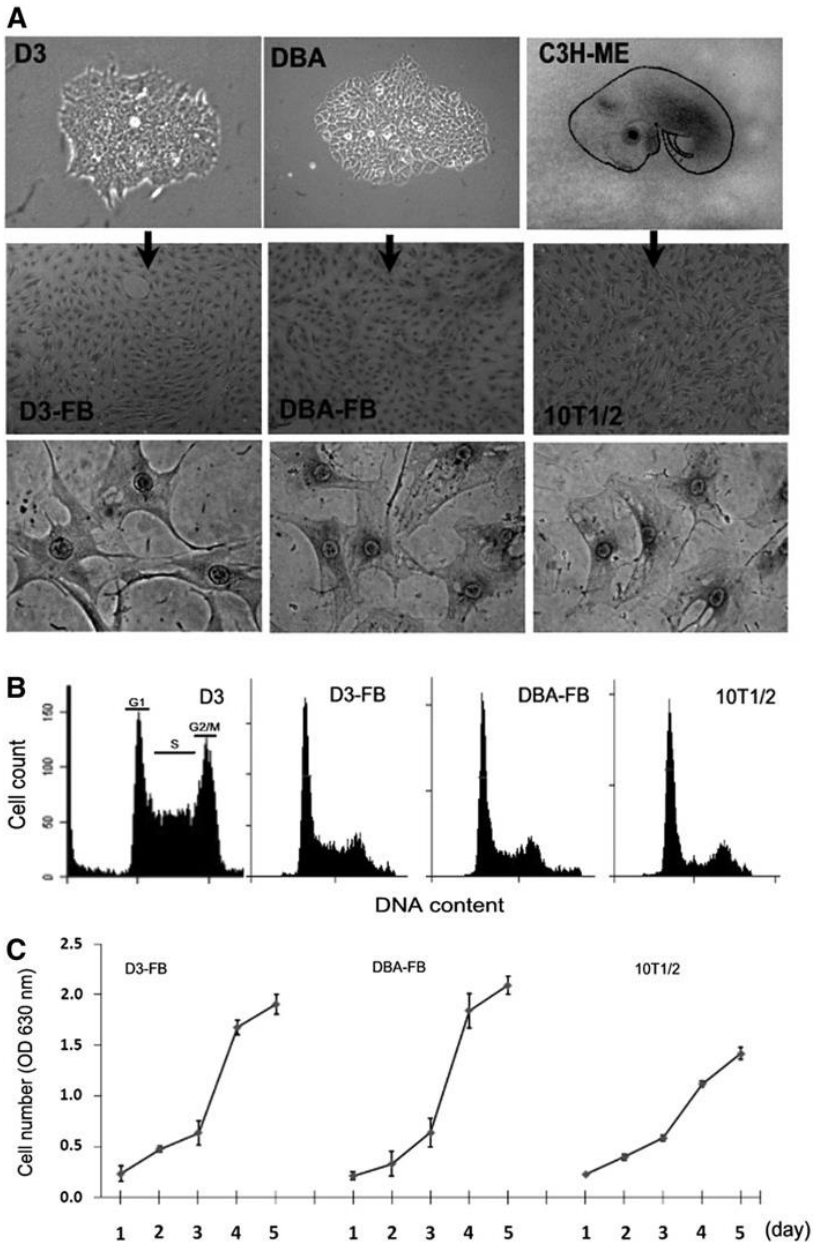

Figure 6. Generation and characterization of mESC-FBs.

(A) Representative process of FB differentiation from two different mESC lines (D3 and DBA) and from C3H embryo. The cells were photographed under a phase contrast microscope. Top panels: colony growth of D3 and DBA (10x); middle panels: purified cells in monolayers (10x, toluidine blue stained); bottom panels: the morphology of single cells (40x, toluidine blue stained). Isolation of 10T1/ 2 from a C3H mouse embryo (C3H-ME) was described by Reznikoff (Pinney and Emerson, 1989; Reznikoff et al., 1973). (B) Cell cycle analysis by flow cytometry. The distribution of cell populations at different phases was denoted in D3 cells. (C) Growth curves of mESC-FBs and 10T1/2. Equal numbers of each cell type were plated at low density (~30% confluence after attachment). Cell proliferation was measured by toluidine blue staining.

Type I IFN expression mechanism is induced, but not effectively, during mESC differentiation

Although mESC-FBs showed considerable similarities to 10T1/2 cells in morphology, growth pattern (Figure 6), and cell marker expression (Wang et al., 2014b), mESC-FBs at early passages (2–10 passages) expressed very low levels of IFNβ (Figure 7A) and IFNα (data not shown) in response to polyIC, which was similar to mESC-DCs (Figure 5B). At the mRNA level, the IFNβ expression capacity of D3-FBs was increased along with their continued in vitro propagation, but it was still substantially lower than 10T1/2 cells (Figure 7A). It is known that signaling molecules that mediate immune responses are usually upregulated by the initial stimulus, forming a positive feedback loop that can in turn boost the cellular responses to subsequent immune challenges (known as the priming effect) (Huang et al., 2006; Matsumoto and Seya, 2008; Pan et al., 2011). Based on this phenomenon, D3-FBs were primed with a low concentration of polyIC (50 ng/mL) for 24 h, a treatment that did not cause detectable cytotoxicity. After 3 days, the primed cells were split and replated in a new cell culture dish, where they were transfected with polyIC (300 ng/mL) a second time. As shown in Figure 7B, the primed cells showed a significantly increased IFNβ mRNA expression compared with unprimed cells in response to the second dose of polyIC stimulation.

The expression of IFNβ and viral RNA receptors in response to polyIC was examined at the protein level by flow cytometry. In 10T1/2 cells, polyIC induced the expression of IFNβ, concurrent with upregulation of three major viral RNA receptors: TLR3, RIG-I, and PKR as indicated by the increased fluorescence intensity (Figure 7C, red lines). The same treatment did not induce the expression of these genes in D3 cells.

However, in D3-FBs (p16), polyIC induced a significantly increased expression of PKR and RIG- I, and to a lesser extent TLR3, but an increase of IFNβ was not detected (Figure 7C). Similar results were obtained in D3-FBs passage 40 (data not shown). It is noted that the basal levels of TLR3, PKR, and RIG-I in control cells (black lines, the dotted lines were set as references) were the lowest in D3 cells. They are increased in D3-FBs, but lower than in 10T1/2 cells. Similar expression profiles for MDA5 were observed (data not shown).We speculate that the failure to detect IFNβ in D3-FBs by flow cytometry could be due to the low sensitivity of this method, which is unlike RT-qPCR that can detect very low- level changes of mRNA. Overall, these results suggested that the differentiation process could induce the IFN expression mechanism, but not to the level in naturally-differentiated 10T1/2 cells. It is noted that transfection efficiency of polyIC is similar among D3, D3-FBs, and 10T1/2 cells as determined by the expression of eGFP from its synthetic mRNA transfected to these cells (data not shown) as previously described (Wang et al., 2014a). Therefore, the different expression levels of IFN in response to polyIC transfection among the three cell types are attributed to their intrinsic properties.

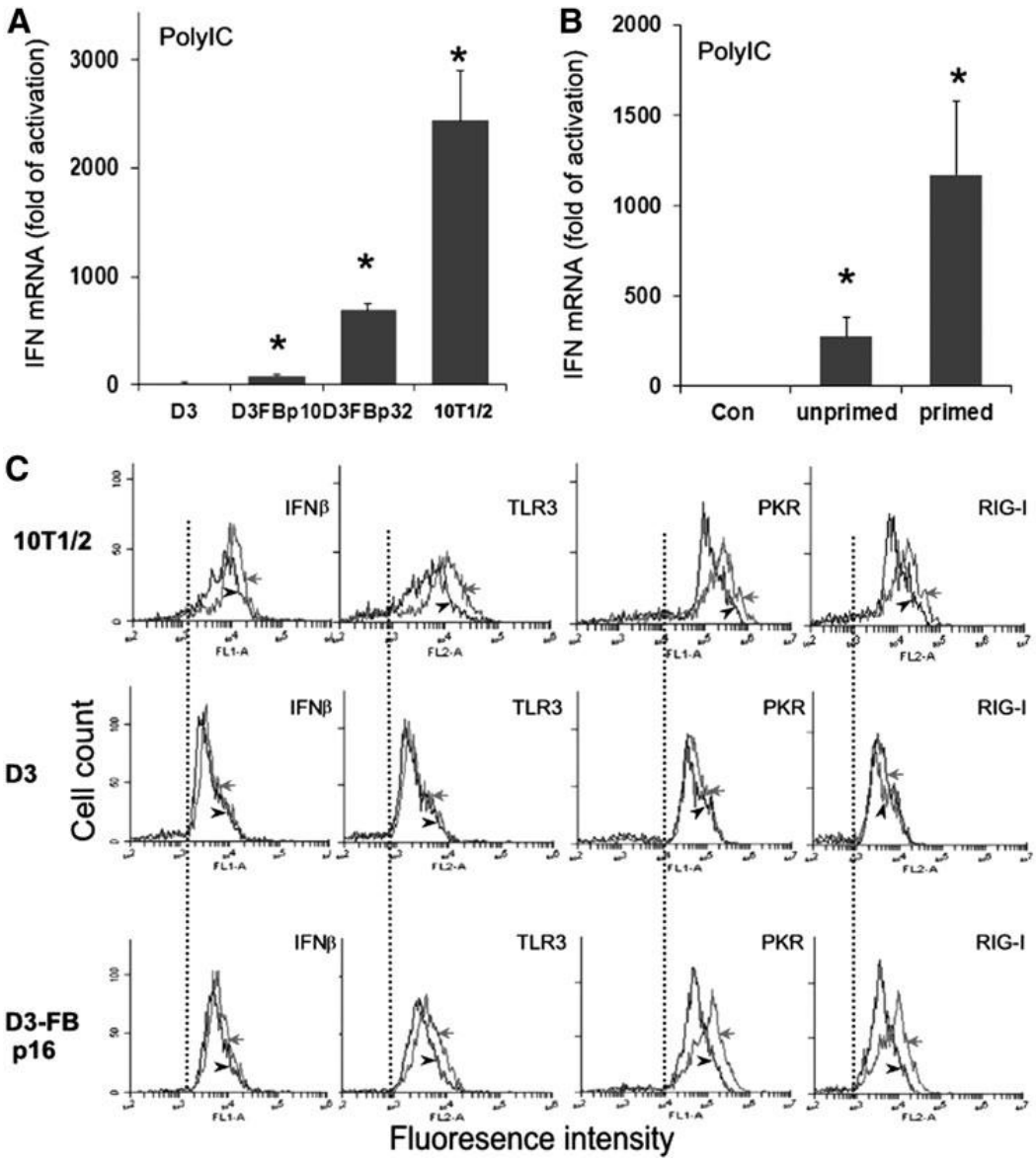

Figure 7. PolyIC-induced IFNβ expression in mESC-FBs and the effect of priming.

(A) PolyIC-induced expression of IFNβ in D3 cells, D3-FBs at different passages (p10 and p32), and 10T1/2 cells. The mRNA levels of IFNβ were determined after the cells were transfected with polyIC for 12 h. The results are expressed as fold activation, where the mRNA level in the control of each set of experiments is designated as 1 (not shown). *P< 0.05, compared with D3 cells. (B) Primed and unprimed D3-FBs (p30-33) (see text for details) were transfected with polyIC (300 ng/mL) for 12 h. The mRNA level of IFNβ was determined under the conditions as described in A. *P<0.05, compared with control cells (Con, without polyIC transfection). (C) PolyIC-induced expression of IFNβ, TLR3, PKR, and RIG-I in 10T1/2 cells, D3, and D3-FBs. The expression levels of the indicated genes were determined by flow cytometry in control cells (lines denoted by arrowheads) and in cells that were treated with polyIC for 20 h (lines denoted by arrows). The dotted lines were used as references to compare the basal levels of the tested genes (in control cells) in different cells (compare vertically).

Upregulation of viral RNA receptors was also observed when mESC-FBs were infected with live viruses. In response to LACV infection, IFNβ expression in early passage D3-FBs (p5) was slightly higher than in undifferentiated D3 cells, but was markedly increased in the cells at passage 50 (Figure 8A). Like polyIC priming, LACV infection also stimulated the expression of viral RNA receptors (Figure 8B). In addition to LACV, we also analyzed the cell response to infection with CHIKV, a virus that is known to effectively infect FBs, and a similar pattern of IFNβ expression to LACV was observed (Figure 8C).

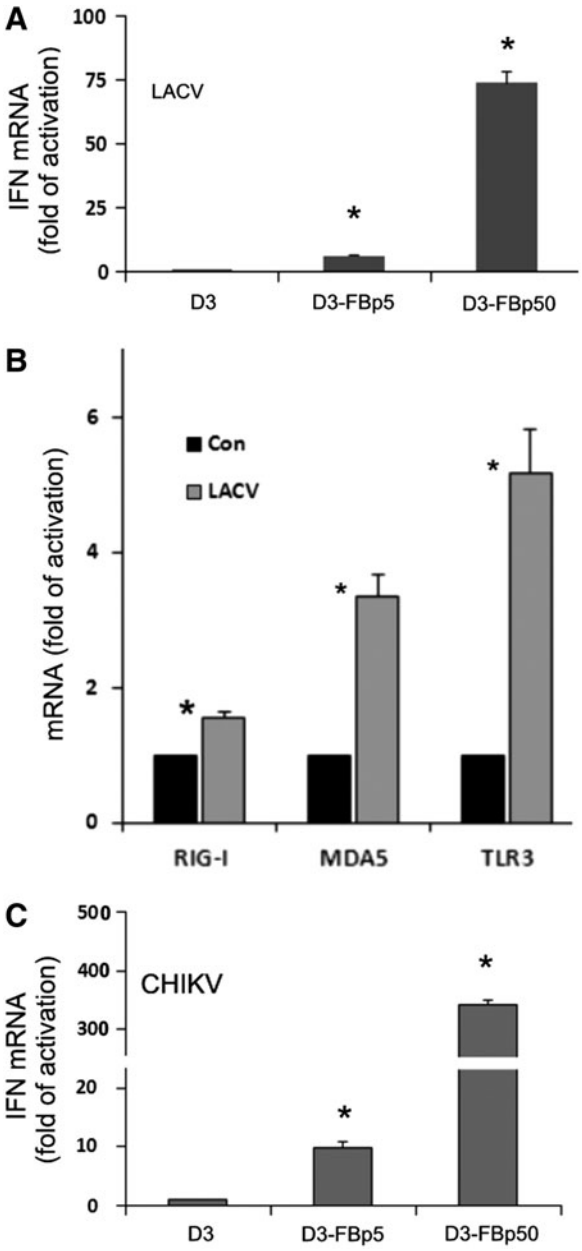

Figure 8. Viral infection-induced expression of IFNβ and viral RNA receptors in mESC-FBs.

D3 cells and D3-FBs at the indicated passages were infected with LACV (A, B) or CHIKV (C). The mRNA levels of IFNβ (A, C) or viral RNA receptors (B, determined from p50) were determined by RT- qPCR at 24h after infection. The results are expressed as fold activation, where the mRNA level in the control of each set of experiments is designated as 1. *P<0.05, (A, C) compared with D3 cells; (B) compared with respective controls (Con).

The NFκB pathway is not functional in mESCs, but is activated in mESC-FBs in response to viral infection

The above results indicated that the molecular mechanism that mediates IFN expression is inducible, although not effectively, by the differentiation process. In seeking the molecular mechanism that underlies this observation, the activation status of NFκB in response to viral infection was examined. NFκB is an essential transcription factor for IFN expression (Kawai and Akira, 2011; Yoneyama and Fujita, 2007). Taking advantage of the dramatic difference in morphology between mESCs and mESC-FBs, a co-culture model was used, where the colony of D3 cells can be easily distinguished from the large flattened D3-FBs in the same dish as illustrated in Figure 9A (Con, phase). The co-cultured D3 and D3- FBs were exposed to CHIKV or LACV under identical conditions. The infected cells were identified by antibodies against a CHIKV or LACV-specific protein (AB3583 or Gc, bright green color). In undifferentiated control cells, NFκB was detected in the cytoplasm of both D3 cells and D3-FBs (Con, red color). Its activation, as indicated by its detection in the nucleus, only took place in virus-infected D3- FBs (CHIKV and LACV, indicated by arrow), but not in virus-infected D3 cells (CHIKV and LACV, indicated by arrowhead). It is noted that uninfected cells, both D3cells and D3- FBs, had their NFκB retained in the cytoplasm (CHIKV and LACV, cells with dark green background) as seen in the control cells. Quantitative analysis of CHIKV-infected cells showed that about 65% of infected D3-FBs were positive for nuclear NFκB staining, whereas essentially no nuclear NFκB was detected in any CHIKV-infected D3 cells (Figure 9B), regardless of the percentage of infected cells in colonies with different sizes. Similar results were observed in cells infected with LACV

(data not shown). The exclusive detection of NFκB in the nucleus of virus-infected D3-FBs clearly demonstrates that the NFκB pathway is functional only after differentiation. The same results were obtained when D3 cells and D3-FBs were cultured separately (data not shown).

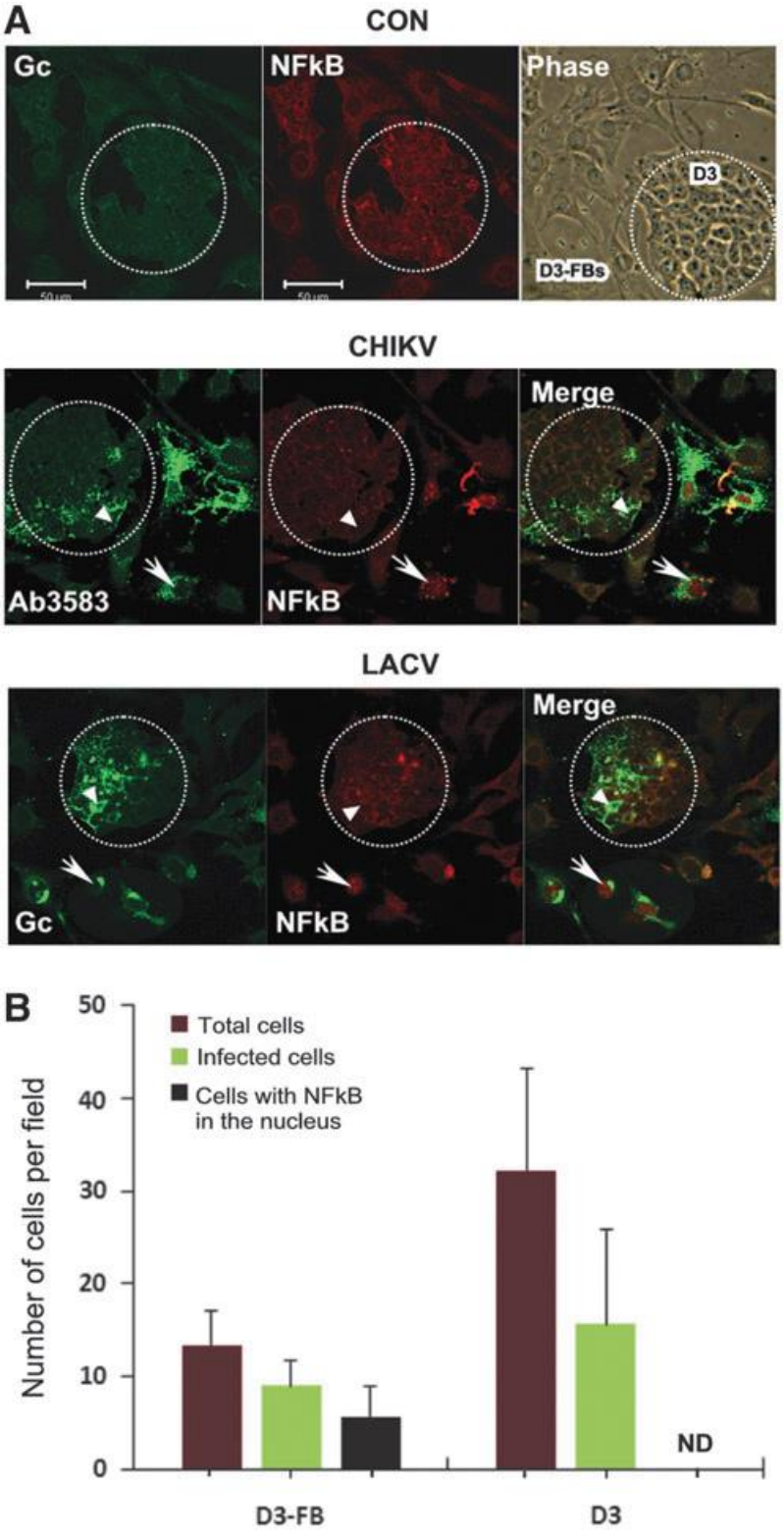

Figure 9. The activation status of NFκB in mESCs and mESC-FBs in response to viral infection.

(A) D3 cells and D3-FBs (p15) were grown in a coculture, where D3 cells were identified by their clonal growth (circled area) and D3- FBs were identified by their flattened large cell bodies under a phase contrast microscope (phase). The cells were infected with CHIKV or LACV for 24h. The cells were double stained with an antibody against NFκB (rabbit) and an antibody against CHIKV (Ab3583, mouse) or an antibody against LACV (GC, mouse). The cellular location of NFκB was detected with a rhodamine-conjugated rabbit secondary antibody (red), whereas the infected cells were identified with FITC-conjugated mouse secondary antibodies (green). The images were acquired under a LSM 510 laser-scanning confocal microscope (scale bar=50μm). In mock control cells (Con), NFκB is mainly detected in the cytoplasm of both D3 cells and D3-FBs. Its activation is indicated by its translocation to the nucleus, which only took place in virus-infected D3-FBs (CHIKV and LACV, indicated by arrows), but not in virus-infected D3 cells (CHIKV and LACV, indicated by arrowheads). The relationship between NFκB localization and cell infection is manifested in the merged images. (B) CHIKV infection-induced NFκB nuclear translocation. The total number of cells was determined from phase contrast images, infected cells were determined from cells expressing the CHIKV protein (Ab3583-positive cells), and NFκB nuclear-positive cells were determined by the detection of NFκB in the nucleus. Data were derived from 10 representative fields for each cell type.

mESC-FBs are susceptible to cytopathic effects of viral infection and are protected by type I IFN

The responses of D3-FBs and 10T1/2 cells to infection with LACV and CHIKV were compared. Both viruses caused cell death as indicated by the reduction of cell number (Figure 10A) and morphological changes (Figure 10B). Cells infected with CHIKV were characterized by the lysis of the cytoplasm, whereas the nucleus was mostly intact until the late stages of cell death (Figure 10B, CHIKV). LACV infection caused a similar cytopathic effect on mESC-FBs, but with less potency (data not shown). Overall, mESC-FBs were less susceptible to the cytopathic effects of CHIKV and LACV than 10T1/2 cells, but they showed the same pattern of lytic cell death at a longer incubation time.

Type I IFN are best characterized for their antiviral activity. We have previously shown that although mESCs cannot express these cytokines (Wang et al., 2013), they could weakly respond to IFN and express IFN-stimulated genes (ISGs). mESCs were

protected from LACV- and CHIKV-induced cell death by exogenously added IFNα, but at a much higher concentration than that needed to protect 10T1/2 cells (Wang et al., 2014b). Therefore, the effects of IFNα on virus-infected mESC-FBs were tested. As shown in Figure 10C, pretreatment with IFNα significantly reduced LACV-induced cell death of mESC-FBs at 10 U/mL. This concentration is about 10 times lower than that needed to protect mESCs from LACV-induced cell death (>100 U/mL) (Wang et al., 2014b). Similar results were observed in CHIKV-infected mESC-FBs (data not shown).

Correlating with the increased ability to mediate the antiviral activity of exogenously added IFNα, D3-FBs express higher mRNA levels of ISG15 than in D3 cells in response to IFNα (Figure 10D, graph). The expression of PKR, another well-characterized ISG (de Veer et al., 2001), was further tested. IFNα induced PKR expression in D3 cells as determined by flow cytometry; however, the induction in D3-FBs is significantly higher than in D3 cells, as judged by the greater increase of fluorescence intensity relative to control cells (red line vs. black lines, Figure 10D, flow profile). Together, these data support the conclusion that mESCs can respond to IFN, but at a much lower level than differentiated cells. However, the IFN response mechanism is further developed in mESC-FBs after differentiation.

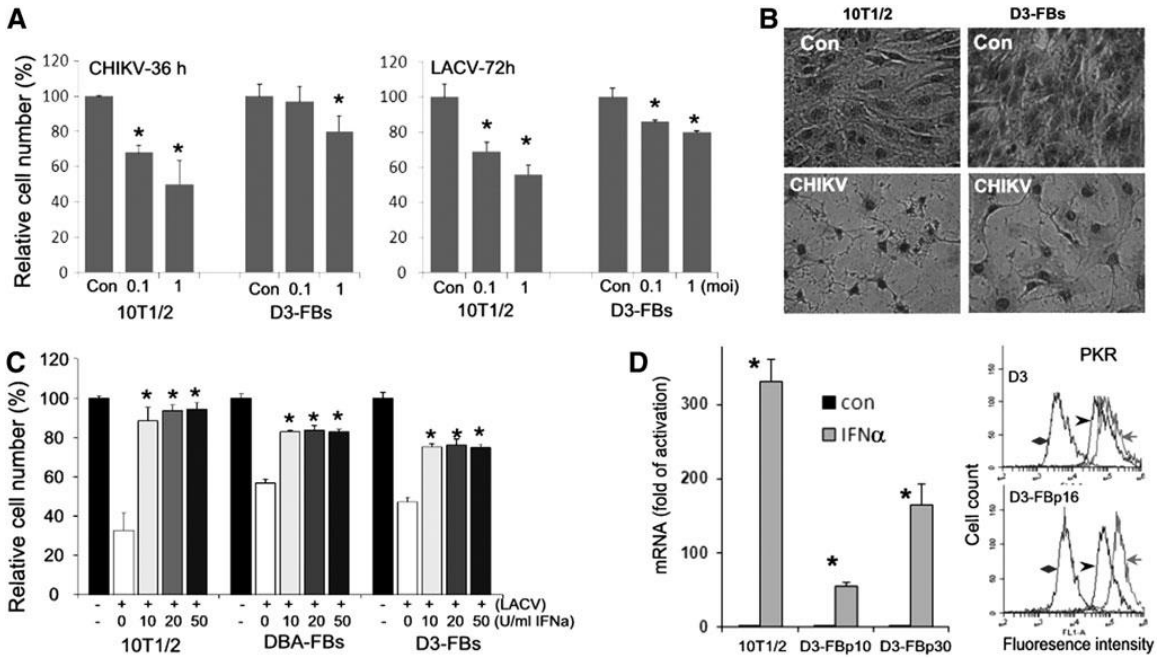

Figure 10. Virus infection-induced cytopathicity and the effect of IFNα.

(A) D3-FBs (p10-25) and 10T1/2 cells were infected with LACV or CHIKV under the specified conditions. The number of cells was determined by toluidine blue staining. The cell numbers in controls (Con) were defined as 100%. *P< 0.05, compared with Con. (B) The morphology of cells was examined under a phase contrast microscope (400x) 36 h post-infection with CHIKV. (C) The 10T1/2 cells and ESC-FBs were pretreated with different concentrations of IFNα for 24 h or left untreated. The cells were then infected with LACV (m.o.i.= 5) for 52 h. The number of viable cells was determined by Toluidine Blue staining. The cell numbers in controls (Con) were defined as 100%. *P< 0.05, compared with infected cells without IFNα pretreatment. (D) Graph, cells were treated with IFNα (500 U/mL) for 12 h. The mRNA levels of ISG15 were expressed as fold activation, where the mRNA level in their respective control cells (without IFN treatment) is designated as 1. *P<0.05, compared with Con. Flow profiles, the expression levels of PKR were determined by flow cytometry in cells treated with IFNα (500 U/mL, 24 h, lines denoted by arrows) and control cells (lines denoted by arrowhead). Lines denoted by diamonds represent cells stained only with FITC-conjugated secondary antibodies as negative controls.

PolyIC-induced cell death of mESC-FBs is mediated by PKR

Previous research from our lab showed that polyIC transfection activated PKR in mESCs and caused cell cycle inhibition, but did not cause apparent cell death (Wang et al., 2013). However, mESC-FBs showed an increased sensitivity to polyIC-induced cell death, similar to 10T1/2 cells, as indicated by the reduced cell number (Figure 11A). Unlike the cell death induced by viral infection, which was attenuated by IFNα treatment

(Figure 10A), polyIC-induced cell death was not affected by IFNα (Figure 11A) or by IFNβ (data not shown). Furthermore, polyIC-induced cell death was characterized by the formation of multiple intracellular vacuoles while the cytoplasm was largely intact at the earlier stage of cell death (Figure 11B). These results indicate that the protecting activity of IFN is limited to the cytopathic effect of viral infection, suggesting that the cell death caused by viral infection and polyIC transfection occurs through different mechanisms.

Activation of PKR has been implicated in polyIC-induced death of somatic cells (Gil and Esteban, 2000). Since polyIC significantly increased the expression of PKR in D3-FBs, but not in D3 cells (Figure 7B), it is likely that the increased PKR expression contributes to polyIC-induced cell death in mESC-FBs. To test this hypothesis, RNAi was used to knock down PKR in mESC-FBs. In a previous study from our lab, it was shown that transfection of mESCs with a cocktail of three different sequence-specific siRNAs against PKR could specifically knock down PKR to a level undetectable by western blot analysis (Wang et al., 2013). The same treatment significantly attenuated polyIC-induced cell death in both D3-FBs and DBA-FBs. A similar result was obtained when the cells were treated with a PKR inhibitor (C16) (Figure 11C). These results suggested that PKR activation is at least partly responsible for polyIC- induced cell death of mESC-FBs.

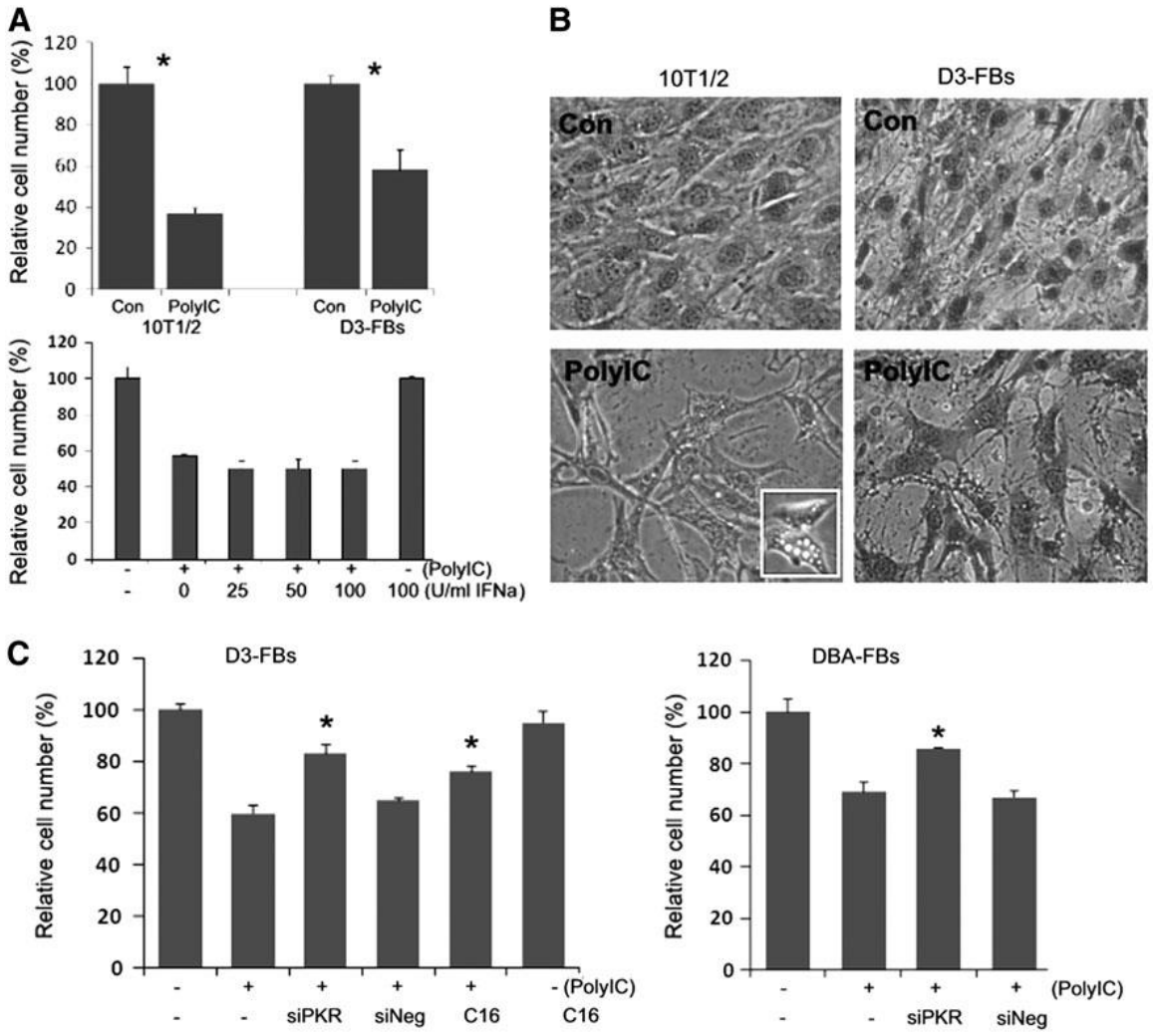

Figure 11. PolyIC-induced cytotoxicity and the role of PKR.

(A), PolyIC-induced cell death was not affected by pretreatment with IFNα. D3-FBs and 10T1/2 cells were transfected with polyIC for 24 h. The cell number was determined by toluidine blue staining. *P<0.05, compared with untreated control (Con). (B), The morphology of cells was examined under a phase contrast microscope (400x). The image inset in polyIC-treated cells show two enlarged individual cells illustrating the intracellular vacuoles. (C), D3-FBs and DBA-FBs were transfected with siRNA against PKR (siPKR) or control siRNA (siNeg) at 100nM for 24 h, or the cells were pretreated with C16 for 30 min, then the cells were transfected with polyIC for 24 h. The number of viable cells was determined by toluidine blue staining. The cell numbers in control experiments (without any treatment) were defined as 100%. *P< 0.05, compared with polyIC-transfected cells. The mESC-FBs used in the above experiments were from passages between 10 and 25 with similar results.

Discussion

The studies described in this chapter have been published in the journal Stem Cells and Development (D'Angelo et al., 2016). The underdeveloped innate immunity has been recently characterized as a unique property of pluripotent cells (reviewed in (Guo et al., 2015)). Since most types of somatic cells have an effective innate immune system, the attenuated immune and inflammatory responses reported in several ESC-derived tissue cells (Földes et al., 2010; Glaser et al., 2011; Rajan et al., 2008; Zampetaki et al., 2006) raise the question of whether the commonly used in vitro differentiation methods can generate cells with competent innate immunity comparable to in vivo-differentiated cells. However, it is not clear whether these findings represent all types of ESC-derived cells or just the observations in the cell types that have been investigated. Furthermore, none of the published studies has investigated antiviral responses under the condition of live viral infection. The results described herein provide valuable insights into the molecular basis for the deficiency of IFN expression in mESCs and the development of antiviral innate immunity during their differentiation.

Differentiation of ESCs through EB formation is the most commonly used method in the literature (Wobus and Boheler, 2005). The results of this study demonstrated that mESC-DCs by this method showed a limited capacity to express IFNβ, which likely represents a common property of multiple cell types since mESC-DCs consist of mixed cell lineages (Guo et al., 2007). C3H 10T1/2 cells were used for comparison for two reasons. First, their embryonic origin makes them particularly suitable since they were FBs derived from 14–17 day old mouse embryos (Pinney and Emerson, 1989; Reznikoff et al., 1973), a time frame similar to in vitro EB

differentiation. Second, FBs are considered to be the major tissue stromal cells that modulate tissue immunity and inflammation since they express large amounts of IFNβ and other inflammatory cytokines in response to immune stimuli (Enzerink and Vaheri, 2011; Jordana et al., 1994). The mESC-FBs generated through RA-induced differentiation share extensive similarities with 10T1/2 cells, but these cells, especially at early passages, displayed a significantly lower capacity to express IFNβ than 10T1/2 cells, similar to mESC-DCs differentiated by EB formation. Therefore, the lack of an effective IFN expression capacity is a major property of mESC-FBs that differs from naturally differentiated 10T1/2 cells. While the mechanism to express type I IFN during differentiation lagged behind the development of other cellular features (i.e. morphology, proliferation rate, cell cycle profile, and cell marker expression), it was nonetheless inducible. The analysis of NFκB in mESCs and mESC-FBs provides an explanation for the deficiency of type I IFN expression in undifferentiated mESCs and the acquisition of its active status in differentiated mESC-FBs. NFκB is an essential transcription factor for the expression of IFN and inflammatory cytokines. Although the functionality of NFκB in ESCs has been an issue of controversy (Armstrong et al., 2006; Torres and Watt, 2008), this study clearly demonstrated its inactive status in virus-infected mESCs, which was also noted by other investigators in mESCs that were treated with TNFα (Kang et al., 2007), the best-studied NFκB activator in differentiated somatic cells. The low expression levels of viral RNA receptors (Wang et al., 2013) (Figure 7C) and the inactive status of NFκB could be major reasons for the attenuated antiviral responses in mESCs. However, the possibility cannot be ruled out that there are other components essential for innate immunity that may not be sufficiently expressed or not functional as well.

The upregulation of viral RNA receptors and activation of NFκB by viral infection in mESC-FBs signifies the acquisition of antiviral innate immunity during differentiation. The data presented in this study suggest that the two commonly used in vitro differentiation methods described in this study can only partly turn on the genes that regulate immune responses. A possible reason for this phenomenon could be that the expression of these genes is not needed because the differentiation takes place under a sterile culture condition. It is known that the expression of immune genes can be upregulated by pathogenic stimuli (Huang et al., 2006; Matsumoto and Seya, 2008; Pan et al., 2011). PolyIC treatment, like acute viral infection, can upregulate the expression of certain immune genes in immune cells and FBs (Huang et al., 2006; Lee et al., 2012). Similar to this finding, polyIC priming induced expression of viral RNA receptors in mESC-FBs, resulting in an increased response to subsequent polyIC transfection. The priming effect of polyIC is likely through a mechanism that was recently described in FBs, by which polyIC induces the expression of multiple innate immune genes through chromatin remodeling (Lee et al., 2012). Similar to polyIC, viral infection also induced the expression of viral RNA receptors. Therefore, we propose that the development of antiviral mechanisms can be accelerated by immunostimuli (i.e. polyIC or viral infection) during ESC differentiation.

As part of the IFNβ-based antiviral mechanism, the capacity to respond to IFN is underdeveloped, but not completely deficient in mESCs (Wang et al., 2014b). The data presented here indicated that the IFN response mechanism is further advanced in mESC-FBs, as indicated by their increased ability to mediate the antiviral effect of IFNα and to induce the expression of ISGs (ISG15 and PKR). It is interesting to note that IFN

treatment showed the expected antiviral activity against virus-induced cell death of mESC-FBs, but was unable to protect the cells from polyIC-induced cell death. While the cell death caused by viral infection is a very complicated process that could involve numerous proteins and is often virus-dependent (Sen, 2001), it is likely that polyIC-induced cytotoxicity in mESC-FBs involved PKR upregulation/activation, which is known to cause cell death of differentiated somatic cells (Gil and Esteban, 2000). We have previously shown that polyIC causes cell cycle inhibition, but not significant cell death (Wang et al., 2013); therefore, the increased sensitivity of mESC-FBs to the toxicity of polyIC is likely due to their higher expression level of PKR than in mESCs in response to both polyIC and IFNα. These results suggest that the differentiation process also promotes the development of cellular mechanisms regulated by the PKR pathway.

Most of the current differentiation methods use certain growth factors or cytokines to promote ESC differentiation toward a particular cell lineage of interest. For example, VEGF and bFGF are commonly used to promote ESC differentiation into vascular endothelial cells (Blancas et al., 2008; Levenberg et al., 2002; McCloskey et al.; Yamashita et al., 2000), whereas PDGF and TGFβ are used for smooth muscle cell differentiation (Sinha et al., 2004; Yamashita et al., 2000). We are unaware of any strategies for the differentiation of structural tissue cells that contain factors with the consideration of innate immunity development. The lack of immunostimulation during in vitro differentiation could be a major reason that accounts for the ineffective development of innate immunity in ESC-derived cells.

The attenuated innate immunity in ESC-derived cells is a complex issue that may have different implications for their therapeutic application. On one hand, it is known that

a strong immune and inflammatory reaction of grafted cells to the host environment could augment the inflammation in the wounded area, therefore negatively impacting tissue repair and healing. In this context, an attenuated innate immunity in ESC-derived cells could be beneficial. On the other hand, the functionality of transplanted ESC-derived cells could be compromised if they do not have a competent innate immunity. For instance, endothelial cells are not only critical for vascular function, but also act as innate immune surveillance cells to sense and combat pathogens in the circulation (Bell, 2009; Mai et al., 2013). Conceivably, the use of ESC-derived endothelial cells for vascular tissue repair could be a concern if they do not have a competent innate immunity (Földes et al., 2010). At the present time, it is not clear to what degree the attenuated innate immunity that I have characterized in ESC-FBs applies to other ESC-derived cell types and how this property may affect their cell type-specific functions, such as the aforementioned endothelial cells. To fully understand these questions, it will be essential to have a complete characterization of ESC-derived cells by in vitro and in vivo studies.

In conclusion, the lack of a functional innate immunity in ESCs has recently attracted much attention as an important subject in basic ESC biology, immunobiology, developmental biology, and regenerative medicine. Based on our previous work and the current study, I conclude that mESCs have underdeveloped antiviral mechanisms; they are deficient in expressing type I IFN, have limited responsiveness to IFN, and have a basic functional PKR pathway. These antiviral mechanisms can be induced during differentiation and could be modulated by the differentiation environment. This knowledge is not only valuable to understand basic ESC biology, but also instructive to

design strategies that can generate ESC-derived cells with desired levels of innate immunity suitable for regenerative medicine.

CHAPTER IV – THE MOLECULAR BASIS FOR THE LACK OF INFLAMMATORY RESPONSES IN MOUSE EMBRYONIC STEM CELLS AND THEIR DIFFERENTIATED CELLS

Introduction

In Chapter III, I demonstrated the development of innate immune responsiveness to viral stimuli during in vitro differentiation of mESCs. In this chapter, the examination of innate immunity is expanded to the inflammatory response, as well as a possible mechanism behind the lack of responsiveness of ESCs.

Recent studies reported that several major tissue cell types differentiated from both human ESCs (hESCs) and mouse ESCs (mESCs) have limited innate immune response to various pathogens and cytokines (Földes et al., 2010; Glaser et al., 2011; Rajan et al., 2008; Sidney et al., 2014; Zampetaki et al., 2006, 2007), highlighting the potential functional deficiency of in vitro ESC-differentiated cells (Guo et al., 2015).

The immunoproperty of ESC-derived cells is an important consideration for their therapeutic application. Immunogenicity, the cause leading to the rejection of implanted cells by the host, has attracted much attention in the studies of ESCs and their differentiated cells (De Almeida et al., 2013; English and Wood, 2011; Tan et al., 2014). In contrast, few studies have investigated their immune and inflammatory responses to the host environment, despite the fact that such responses significantly impact the outcome of transplantation. The effects of the lack of innate immunity in ESC-differentiated cells remain to be evaluated; however, it is likely that this deficiency may affect their fate and functionality when used in a clinical setting, because potentially they will be placed in an inflammatory area of the patient. Although the attenuated innate

immunity in ESC-differentiated cells may compromise their contribution to the tissue immunity, it could also be beneficial because the implanted cells would not potentiate the inflammatory response in the wounded area, thus avoiding further damage caused by the host's adaptive immunity (English and Wood, 2011). Therefore, determining the molecular mechanisms that control innate immunity development and the immunoproperties of ESC-differentiated cells will provide valuable information for evaluating their therapeutic potential.

Innate immunity, presumably developed in most, if not all mammalian cells, is considered to be the first line of an organism's defense and plays a critical role in mobilizing adaptive immunity. The cellular response to viral/bacterial pathogens and inflammatory cytokines is the central part of innate immunity. The lack of such function in ESC-derived cells raised concerns about their therapeutic application, and it promoted studies seeking the molecular mechanisms in ESCs from which these cells are derived. Indeed, it was demonstrated that ESCs do not show immune responses typically seen in differentiated cells infected with bacteria and viruses (Wash et al., 2012; Yu et al., 2009). Our recent studies in mESCs (Wang et al., 2013, 2014a, 2014b) and those by other investigators in hESCs and in induced pluripotent stem cells (Chen et al., 2012, 2010) demonstrated that the IFN system, the central component of innate antiviral immunity in differentiated somatic cells (Samuel, 2001), is not fully developed in these cells. Therefore, the lack of innate immune responses to bacterial and viral infection appears to be an intrinsic property of all pluripotent stem cells (Guo et al., 2015).

The cellular immune response is induced by various products from microbial pathogens. Immunostimuli are mainly detected by pattern recognition receptors that

include TLRs and retinoic acid-inducible gene I-like receptors (Kawai and Akira, 2011; Yoneyama et al., 2004). Although different immunostimuli are detected by distinct receptors, and the signals are transduced by different signaling pathways, the signal transduction eventually converges at the point of NFκB activation. Activated NFκB, alone or together with other transcription factors, directly controls the transcription of IFN, inflammatory cytokines, and many other types of inflammatory mediators (Kato et al., 2011; Kawai and Akira, 2011). Therefore, the activation of NFκB plays a central role in immune and inflammatory responses. In mammals, the NFκB family is composed of five related transcription factors: p50, p52, RelA, c-Rel, and RelB. They activate transcription of target genes through hetero- or homodimerization. The canonical NFκB pathway involves p50/RelA (or c-Rel) and is mainly activated by pathogens and inflammatory cytokines, whereas the noncanonical pathway uses p52/RelB and is usually activated by specialized factors (Hayden and Ghosh, 2012). Previous studies indicated that the lack of IFN expression in ESCs is attributable, in part, to the absence or low levels of expression of viral RNA receptors (Chen et al., 2010; Wang et al., 2013). Our recent study further demonstrated that NFκB is not activated in virus-infected mESCs, which explains the deficiency in IFN expression at the transcriptional level (D'Angelo et al., 2016).

Although significant progress has been made in understanding the lack of antiviral innate immunity in mESCs and hESCs, little is known about the antibacterial and inflammatory responses in these cells. Because the NFκB pathway is commonly activated by various pathogens and inflammatory cytokines, it was reasoned that its inactive state in ESCs could also account for their lack of antibacterial and inflammatory

responses. However, a limited number of published studies reported different results with inconsistent conclusions (Guo, 2016). In this study, it is demonstrated that mESCs and hESCs are deficient in mounting inflammatory responses to LPS (a bacterial endotoxin that strongly induces inflammatory response), TNFα (a prototypical inflammatory cytokine), and viral infection. In vitro differentiation can induce, but only partially, the development of the inflammatory response mechanism in mESC- differentiated cells (mESC-DCs). Here the molecular basis underlying these observations is provided.

Methods

Cell treatment

ESCs (30–50% confluence), mESC-FBs, and other cells (70–80% confluence) were treated with LPS (1 mg/mL; Sigma), human or mouse TNFα or IL-1β (20 ng/mL; PeproTech), or actinomycin D (ActD; 0.4 mg/mL; Sigma) under the conditions specified in the individual experiments.

Flow cytometry

Protein analysis by flow cytometry was performed as described in Chapter III. Anti-TLR4 and CD14 Abs were preconjugated with PE (BioLegend). Anti-RelA antibodies (Santa Cruz Biotechnology) were detected with secondary antibodies that were conjugated with FITC. Isotype antibodies or cell samples without primary antibody incubation were used as negative controls and were used as controls for fluorescence gating.

TNFα cytotoxicity analysis

The cells were treated with TNFα or ActD alone, or they were treated with ActD for 30 min, followed by treatment with TNFα for the specified times. Cell viability was measured by TB staining as described in the Methods section of Chapter III.

Immunocytochemistry

Immunocytochemistry was performed as described in the Methods section of Chapter III. The antibodies used in this study were purchased from Santa Cruz Biotechnology (RelA subunit of NFκB, IkB, and α-SMA) and BD Biosciences (PECAM1).

Reverse transcription real-time polymerase chain reaction

Reverse transcription real-time polymerase chain reaction was performed as described in the Methods section of Chapter III. The sequences of the primer sets for human genes are listed in Table 2.

Table 2

RT-qPCR primer sequences for human genes

Gene	Sequence (forward)	Sequence (reverse)
β-actin	CATGTACGTTGCTATCCAGGC	CTCCTTAATGTCACGCACGAT
ICAM1	AGAGGTCTCAGAAGGGACCG	GGGCCATACAGGACACGAAG
IL-6	AACCTGAACCTTCCAAAGATGG	TCTGGCTTGTTCCTCACTACT

mESC differentiation, reverse transcription real-time quantitative polymerase chain reaction (RT-qPCR), and statistical analysis were described in the Methods section of Chapter III.

Results

LPS, TNFα, and IL-1β fail to activate NFκB in mESCs

In resting cells, NFκB is retained in the cytoplasm by binding to IkB. Upon cell activation, IkB is degraded, and NFκB translocates to the nucleus where it activates transcription of target genes. Therefore, nuclear translocation is commonly used as an indicator of NFκB activation (Guo et al., 1999). The response of NFκB to LPS, TNFα, and IL-1β, three well-known agents that induce the expression of inflammatory genes through the activation of NFκB, was investigated. As shown in Figure 12A, mESCs (DBA and D3) are characterized by their small size with a large nucleus and clonal growth. In control cells, NFκB was detected in the cytoplasm of mESCs, as expected. However, treatment with LPS, TNFα, or IL-1β did not cause a detectable change (Figure 12A). In contrast, TNFα induced clear translocation of NFκB from the cytoplasm to the nucleus in HUVECs, which were used as a positive control (Rajan et al., 2008) (Figure 12B, upper panels), concurrent with complete degradation of IkB, which did not take place in DBA cells (Figure 12B, lower panels) or D3 cells (data not shown). The three agents did not cause detectable changes in NFκB cellular location with 15–90 min of treatment, indicating that none of them could activate the NFκB pathway in mESCs.

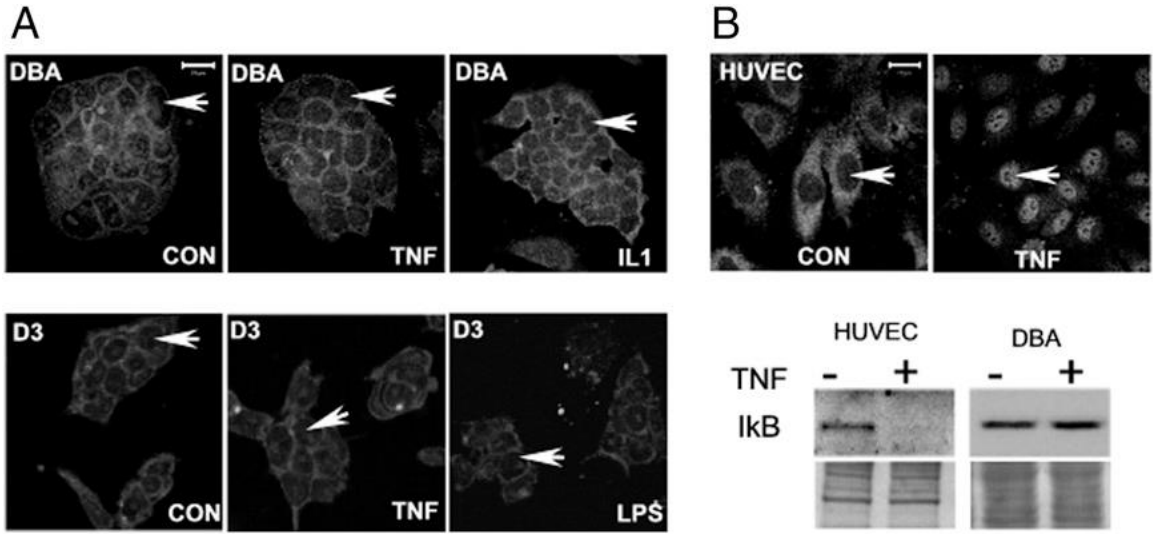

Figure 12. NFκB is not activated by TNFα, IL-1β, or LPS in mESCs.

(A) mESCs (D3 and DBA) were treated with TNFα, IL-1β, or LPS for 20 min or were left untreated (CON). The cellular location of NFκB was analyzed with an antibody against NFκB (RelA subunit) under a fluorescence microscope. (B) TNFα-induced NFκB nuclear translocation in HUVECs (upper panels). Cells were treated and analyzed for the cellular location of NFκB under the conditions described in (A). HUVECs and DBA cells were treated with TNFα for 20 min, and the level of IkB was analyzed by Western blot (lower panels). The lower portions of the blots show the proteins stained with Ponceau S to show the loading of the protein samples. Scale bars, 20 mm. Arrows indicate the location of a representative nucleus. The images are from representative experiments performed at least two times.

TNFα, but not LPS, induces limited activation of NFκB in mESC-DCs and mESC-FBs

It was hypothesized that the lack of NFκB activation could be an intrinsic property of mESCs. Thus, differentiation should turn on the NFκB pathway and the developmental program that controls the inflammatory response. The pluripotency of mESCs is maintained by LIF. Removal of LIF from the culture medium triggers spontaneous differentiation (Niwa et al., 1998). When mESCs are cultured in suspension, they grow in aggregates and form EBs, three-dimensional structures that resemble an early embryo (Guo et al., 2007). As shown in Figure 13Aa, when EBs (inset) were allowed to attach to the surface of a cell culture dish, the cells in EBs differentiated into a round patch-like structure in which cells undergo further differentiation to different cell

types (collectively designated as ESC-DCs) (Guo et al., 2007). Figure 13Aa illustrates a section of this patch structure in which cells can be roughly divided into three zones: the cells in the center zone (Z1) are highly compacted with small size, representing the least differentiated cells; the cells in the middle zone (Z2) are heterogeneous in morphology; and the cells at the peripheral zone (Z3) are large, flattened cells forming a relatively uniform monolayer.

To test the activation of NFκB, ESC-DCs were exposed to TNFα and LPS under the conditions described in Figure 12. Treated cells were double stained with an antibody against NFκB and an antibody against PECAM1 (an endothelial marker) or an antibody against α-SMA (a marker for smooth muscle cells/fibroblasts). TNFα or LPS did not induce NFκB nuclear translocation in the cells in zone 1 (Figure 13Ab, zone 1, a representative nucleus is denoted by an arrow) or the cells in zone 2, including the endothelial cells that were assembled into prototypes of vessel-like structures (Figure 13Ab, zone 2). However, TNFα, but not LPS, induced detectable NFκB nuclear translocation in the cells in zone 3, where the majority of the cells are large, flattened α-SMA+ cells (Figure 13Ab, zone 3). These cells can be easily separated from other cell types in mESC-DCs based on their plastic-adhering property. TNFα-induced nuclear translocation of NFκB was confirmed in the purified α-SMA+ cells (Figure 13Ac).

NFκB activation was then examined in mESC-FBs, which were derived from mESCs through retinoic acid-induced differentiation. They share extensive similarities with 10T1/2 cells (fibroblasts isolated from a 14-d-old embryo) (Pinney and Emerson, 1989; Reznikoff et al., 1973) with regard to cell marker expression, growth pattern, and morphology, as described previously (D'Angelo et al., 2016; Wang et al., 2014b). TNFα-

induced NFκB nuclear translocation is clearly demonstrated in D3-FBs, although the fluorescence intensity of NFκB in their nuclei is substantially lower than in 10T1/2 cells (Figure 13Ba). This result indicates that NFκB has undergone the transition from an inactive status in mESCs to an active status in mESC-FBs. For a direct comparison, a co-culture model of D3 and D3-FBs (or 10T1/2 cells) was used, where D3 colonies could be easily distinguished from the large, flattened D3-FBs or 10T1/2 cells under either a phase-contrast or fluorescence microscope (Figure 13Bb, Figure 13Bc, respectively, circled cells). In control cells, NFκB was detected in the cytoplasm of D3 cells and D3-FBs or 10T1/2 cells. TNFα-induced NFκB nuclear translocation took place in D3-FBs and 10T1/2 cells but not in D3 cells (Figure 13Bc, indicated by an arrow in D3-FBs or 10T1/2 cells and by an arrowhead in D3 cells). The exclusive detection of NFκB in the nucleus of D3-FBs clearly demonstrated that the NFκB pathway is activated only after differentiation. A similar observation was made in cells that were treated with IL-1β, but LPS did not induce NFκB nuclear translocation in D3 cells, D3-FBs, or 10T1/2 cells (data not shown).

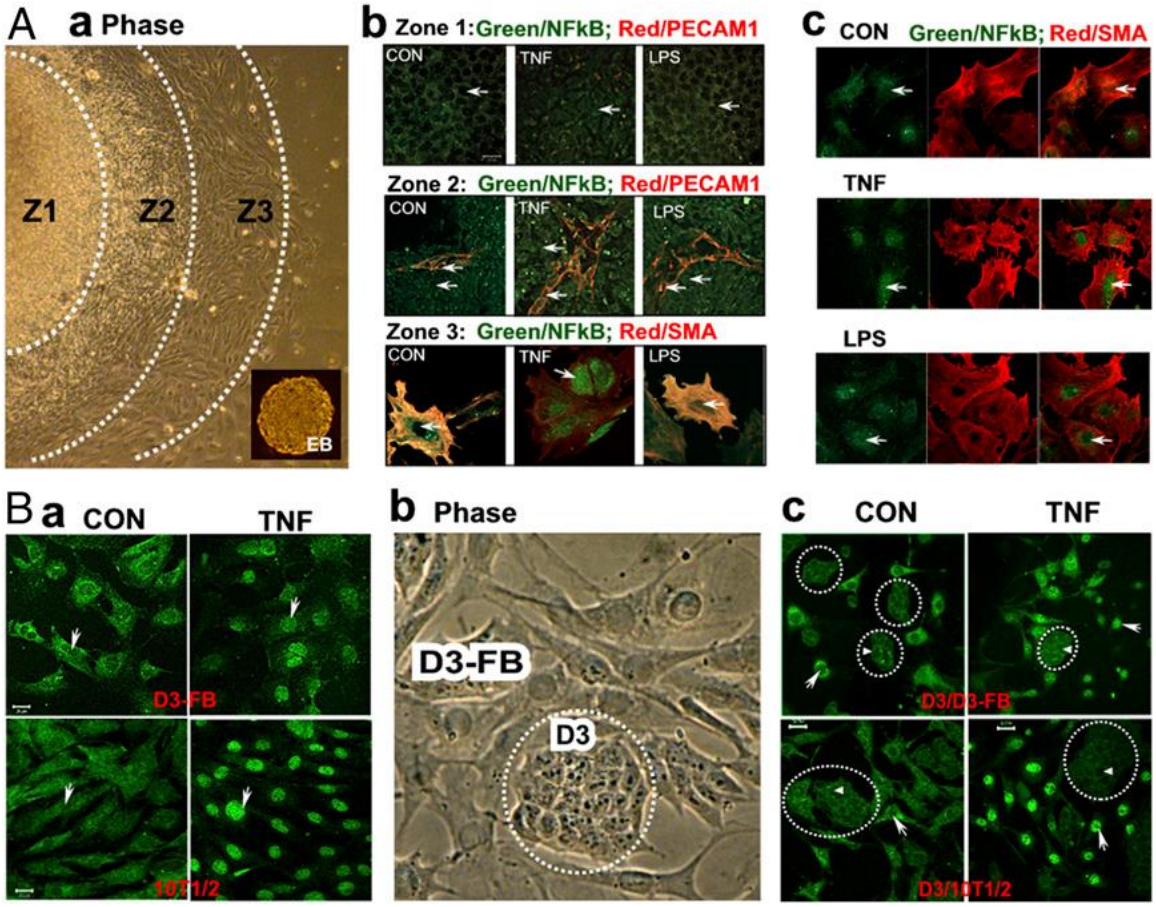

Figure 13. Differentiation of mESCs and the effects of TNFα and LPS on NFκB activation in mESCs and their differentiated cells.

(A) Differentiation of mESC-DCs through EB formation. (Aa) The morphology of mESC-DCs generated from an EB (inset) located in different zones (Z1, Z2, and Z3). (Ab) Immunolocalization of NFκB (green), endothelial cells (PECAM1, red), and α-SMA+ cells (SMA, red) in different zones. (Ac) Purified SMA+ cells were double stained with antibodies against NFκB (green) and SMA (red). In (Ab) and (Ac), the cells were treated with TNFα for 15 min or LPS for 30 min. An arrow indicates the location of a representative nucleus. (B) TNFα induced activation of NFκB in mESC-FBs but not in mESCs. (Ba) D3-FBs and 10T1/2 cells were treated with TNFα for 20 min. The cellular location of NFκB was analyzed with antibodies against RelA subunit. An arrow indicates the location of a representative nucleus. (Bb) D3 cells and D3-FBs (or 10T1/2 cells, not shown) were grown in a co-culture in which D3 cells were identified by their clonal growth (circled area) and D3-FBs were identified by their flattened large cell bodies under a phase-contrast microscope. (Bc) The cells in the co-culture were treated with TNFα and analyzed for the cellular location of NFκB, as described in (Ba). In CON, NFκB is mainly detected in the cytoplasm of all cells tested. TNFα-induced NFκB nuclear translocation took place in D3-FBs and 10T1/2 cells [(Ba) and (Bc), arrows] but not in D3 cells [(Bc), arrowheads]. Scale bars, 20 mm. The images are from representative experiments performed at least two times. CON, control cells that were not treated.

Effects of TNFα and LPS on the expression of inflammatory genes in mESCs, mESC-DCs, and mESC-FBs

The responsiveness of mESCs and their differentiated cells to LPS and TNFα was further analyzed by determining the expression of ICAM1 and IL-6, two genes that are known to be under the transcriptional control of NFκB (Ledebur and Parks, 1995; Libermann and Baltimore, 1990). As shown in Figure 14A, neither LPS nor TNFα induced the expression of the two genes in D3 cells. TNFα induced a slight increase in ICAM1 and IL-6 in mESC-DCs (2–5-fold) after 12 h of incubation. The effect of TNFα was notably increased in mESC-FBs, in which the expression of ICAM1 and IL-6 was induced ~4- and 20-fold, respectively, but it was substantially lower than the effect of TNFα in 10T1/2 cells at all time points tested (Figure 14A, 14B). LPS failed to induce expression of the two genes in D3 cells, D3-FBs, and even 10T1/2 cells (Figure 14A, 14B). Similar results were obtained when the cells were treated with TNFα (up to 100 ng/mL) and LPS (up to 10 μg/mL) (data not shown). To confirm this observation, the effect of LPS was tested on RAW cells and HUVECs as positive controls. LPS induced the expression of ICAM1 and IL-6 in both cells, as expected (Figure 14C). The expression levels of ICAM1 and IL-6 in D3 cells, D3-DCs, D3-FBs, and 10T1/2 cells correlated well with NFκB nuclear translocation in response to LPS and TNFα (Figs. 12, 13). Together, the data suggest that the differentiation process induced, but only partially, the development of the molecular mechanisms that mediate the inflammatory response.

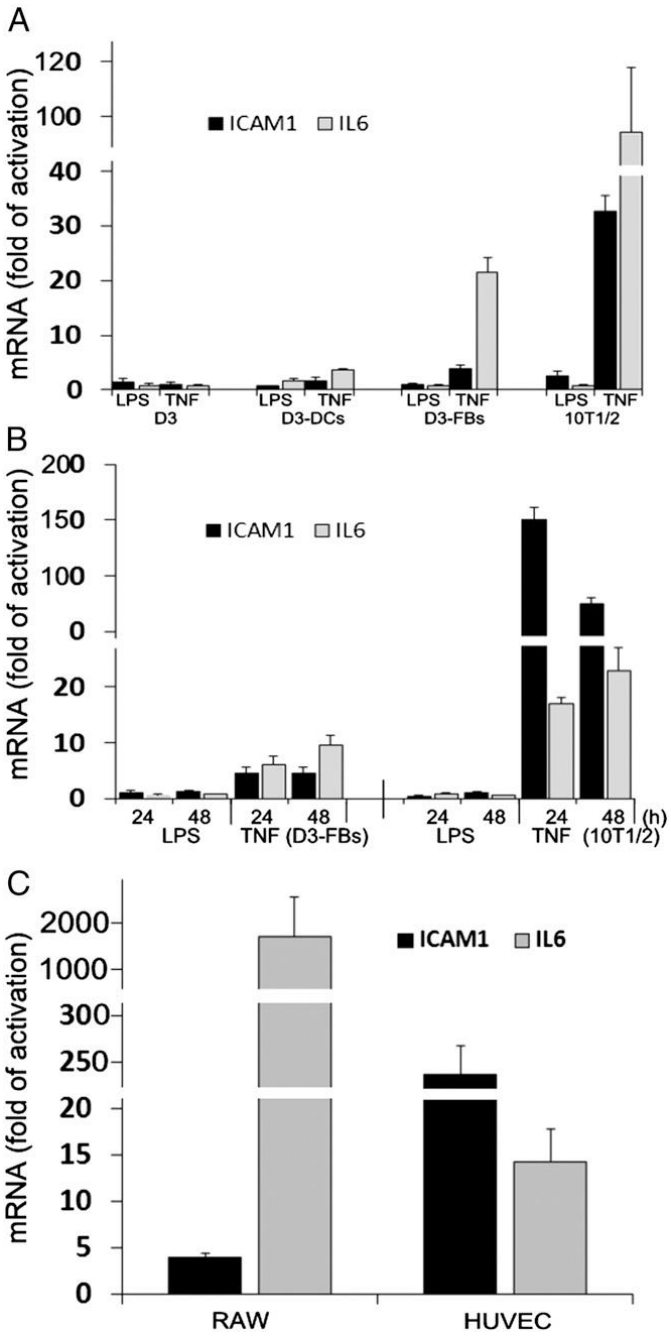

Figure 14. TNFα- and LPS-induced expression of ICAM1 and IL-6 in mESCs and mESC-DCs.

Cells were treated with TNFα and LPS for 12 h (A) or for 24 and 48 h (B). 10T1/2 cells were used for comparison. To confirm the effect of LPS, RAW cells and HUVECs were treated with LPS (5 and 12 h, respectively). (C) The expression of ICAM1 and IL-6 was determined by RT-qPCR. The results are expressed as fold-activation; the mRNA level in the control of each set of experiments is designated as 1 (not shown). The values are mean ± SD of representative experiments performed in triplicate that were repeated at least three times.

LPS and TNFα do not activate NFκB and are unable to induce the expression of inflammatory genes in hESCs

To determine whether the observations made in mESCs also apply to hESCs, the effects of LPS and TNFα on hESCs were analyzed under similar conditions as described for mESCs. As shown in Figure 15 (images), TNFα strongly induced NFκB nuclear translocation in HeLa cells as a positive control, but it failed to induce any detectable change in hESCs. A similar observation was made in hESCs that were treated with LPS (data not shown). In response to LPS and TNFα, HeLa cells, but not hESCs, expressed ICAM1 and IL-6 (Figure 15, graph). These results are basically the same as the observations made in mESCs (Figures 12, 14), indicating that the lack of an inflammatory response to LPS and TNFα is a common feature of mESCs and hESCs.

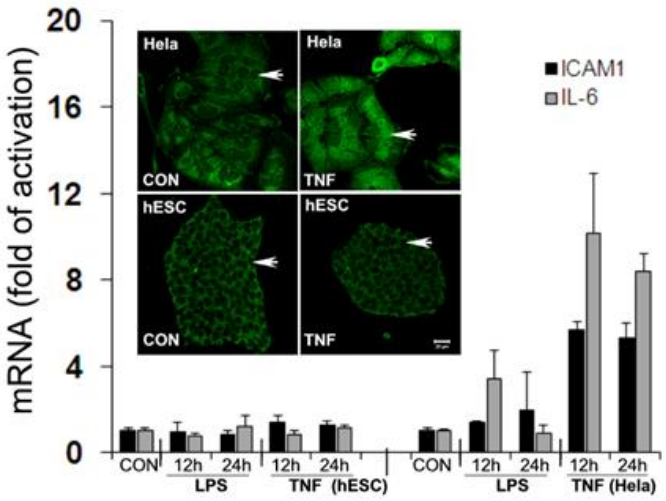

Figure 15. NFκB is not activated by TNFα or LPS in hESCs.

hESCs and HeLa cells were treated with TNFα or LPS for 20 min or were left untreated (CON). The cellular location of NFκB was analyzed with an antibody against NFκB (RelA subunit) under a fluorescence microscope (inset). An arrow indicates the location of a representative nucleus. Scale bar, 20 mm. The bar graph shows hESCs and HeLa cells that were treated with TNFα and LPS for 12 and 24 h. The expression of ICAM1 and IL-6 was determined by RT-qPCR. The results are expressed as fold activation; the mRNA level in untreated control cells (CON) is designated as 1. The values are mean ± SD of a representative experiment performed in triplicate that was repeated two times.

mESC-FBs, but not mESCs, are sensitive to TNFα cytotoxicity

TNFα is a pleiotropic cytokine that is involved in inflammation, as well as causes cytotoxicity that leads to apoptosis or necrosis of certain tumor cells or infected cells (Sedger and McDermott, 2014). A different approach was taken to further investigate the responsiveness of mESCs and mESC-FBs to TNFα. Normal tissue cells are usually resistant to the cytotoxicity of TNFα, but they become susceptible when they are exposed to TNFα in the presence of transcription or translation inhibitors (Guo et al., 1998, 1999). TNFα alone (10–20 ng/mL) did not show detectable toxicity in D3 cells, D3-FBs, or 10T1/2 for up to a 3 day incubation period, but it caused significant cell death of L929 cells (a fibroblast cell line that is sensitive to TNFα toxicity) within a 24 h incubation (data not shown). However, when the cells were treated with TNFα in the presence of the transcription inhibitor ActD, 10T1/2 cells quickly lost viability after 5–6 h of incubation, and ~90% of cells lost viability by 12 h. D3-FBs were susceptible to the cytotoxic effect but with less sensitivity (Figure 16). ActD alone caused apparent cell death of 10T1/2 and D3-FBs at 12 h. D3 cells were more sensitive to ActD than D3-FBs and 10T1/2 cells; toxicity was apparent as early as 6 h. However, a major difference is that, unlike in 10T1/2 and D3-FBs, TNFα did not exacerbate the toxicity caused by ActD in D3 cells (Figure 16B, ActD versus ActD+TNF). These results provide additional evidence that the mechanisms to detect and mediate the effects of TNFα are not developed in mESCs but are promoted by the process of differentiation. The higher sensitivity of D3 cells to ActD toxicity is likely due to their rapid cell proliferation and metabolic rate that depend on the transcriptional activity.

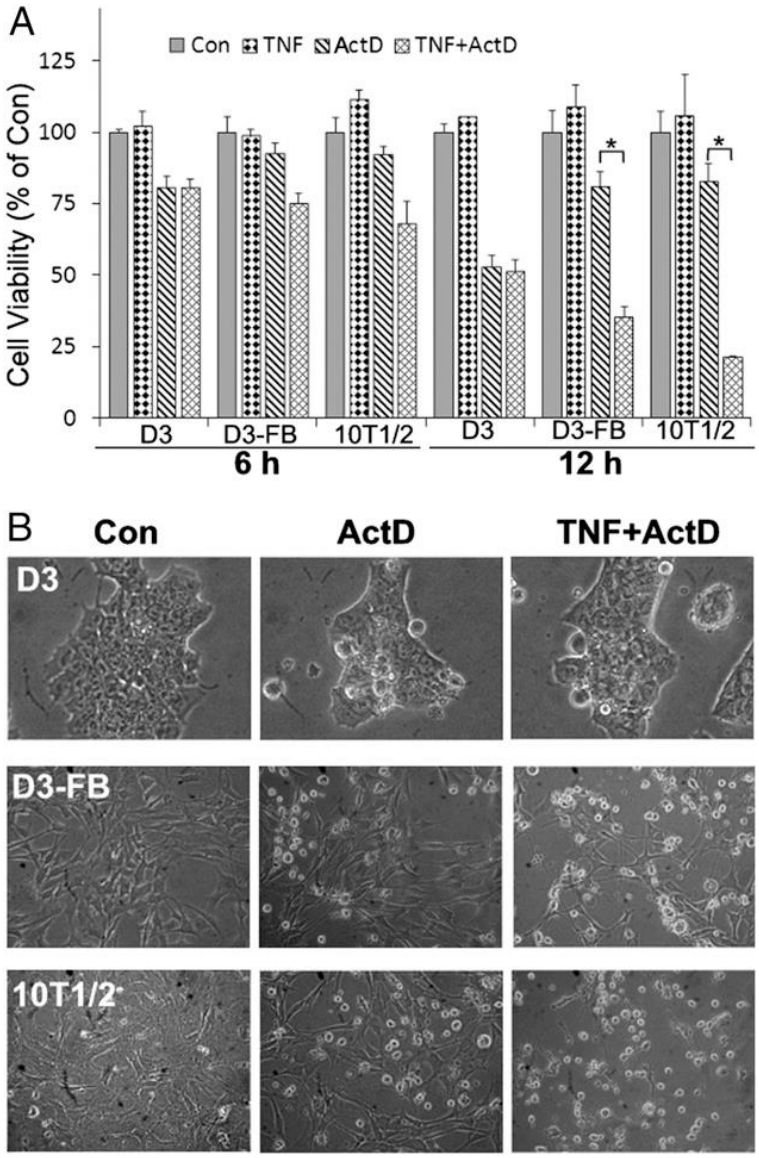

Figure 16. mESCs are insensitive to TNFα cytotoxicity.

D3 cells, D3-FBs, and 10T1/2 cells were incubated with ActD for 30 min, followed by treatment with TNFα for the indicated times. (A) Cell viability was determined by toluidine blue staining. Data are mean ± SD of a representative experiment performed in triplicate that was repeated at least three times. (B) The morphology of cells was examined at 12 h of treatment and photographed under a phase-contrast microscope (original magnification x 400). *$p < 0.05$.

Viral infection induces inflammatory molecules in mESCs and mESC-FBs

The IFN response is the central part of the antiviral mechanism, but the inflammatory response is also a prominent event associated with viral infection. We

showed previously that mESCs do not express IFN when infected with several viruses (D'Angelo et al., 2016; Wang et al., 2014b), including CHIKV, which causes an inflammatory response in a mouse model and in cultured cells, as reported recently (Acharya et al., 2015). D3 cells, D3-FBs, and 10T1/2 cells were infected with CHIKV andthe expression of several inflammatory molecules was analyzed. As illustrated in Figure 17A, CHIKV infection induced the expression of TNFα, ICAM1, and MCP5 in 10T1/2 cells but not in D3 cells. CHIKV-infected D3-FBs expressed increased levels of these molecules compared with D3 cells, but at levels much lower than in 10T1/2 cells. These results resemble the pattern of IFN expression in those cells that were infected with CHIKV (D'Angelo et al., 2016; Wang et al., 2014b).

Because inflammatory cytokines play important roles in the regulation of antiviral responses, the effects of LPS and TNFα on the viability of CHIKV-infected cells were further tested. D3 cells, D3-FBs, and 10T1/2 cells were treated with LPS and TNFα under the conditions described in Figure 16, followed by infection with CHIKV. As shown in Figure 17B, CHIKV infection caused apparent cell death in all three types of cells; however, TNFα augmented CHIKV-induced cell death in D3-FBs and 10T1/2 cells but not in D3 cells (Figure 17B, upper panel), whereas LPS did not show an additional effect on CHIKV-induced cell death in any cells tested (Figure 17B, lower panel). These results further support the conclusion that mESCs are unable to mount an inflammatory response. Differentiated mESC-FBs have partially developed the mechanism to respond to TNFα, but they lack the mechanism to respond to LPS.

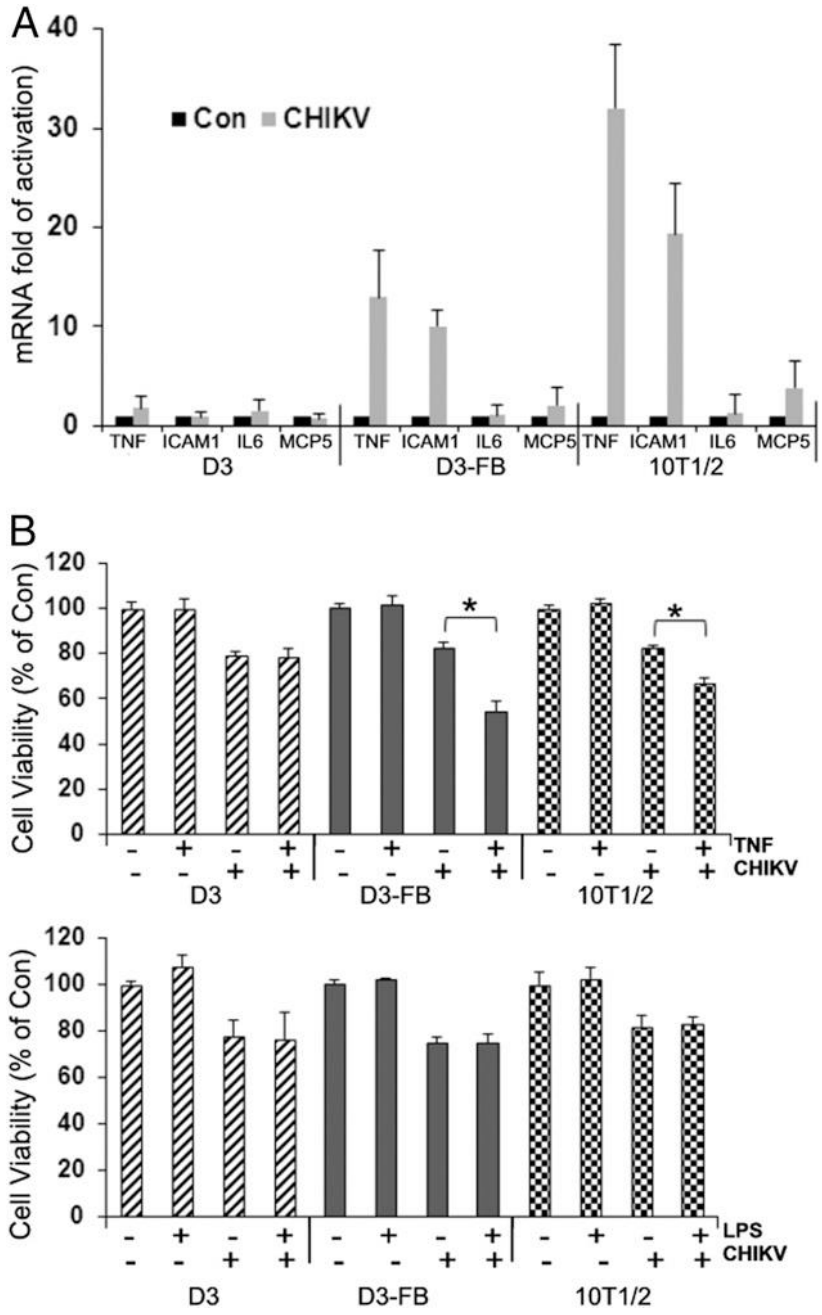

Figure 17. Viral infection-induced expression of inflammatory molecules in mESCs and mESC-FBs and the effect of LPS and TNFα.

(A) D3 cells, D3-FBs, and 10T1/2 cells were infected with CHIKV (MOI = 1) for 20 h. The mRNA levels of tested genes were determined by RT-qPCR and compared with their mRNA levels in cells without infection (Con, designated as 1). (B) D3 cells, D3-FBs, and 10T1/2 cells were incubated with TNFα (upper panel) or LPS (lower panel) for 3 h, followed by CHIKV infection for 20 h. Cell viability was determined by toluidine blue staining. Data are mean ± SD of a representative experiment performed in triplicate that was repeated two times. *$p < 0.05$.

Increased expression levels of LPS and TNFα receptors and NFκB during differentiation

To define the molecular basis underlying the attenuated inflammatory responses in mESCs and mESC-FBs, the expression levels of TLR4 and CD14, the receptor and co-receptor for LPS, respectively, were analyzed by flow cytometry. TLR4 was not detected in D3 cells and D3-FBs, whereas the expression of CD14 in D3-FBs was slightly higher than in D3 cells (Figure 18A). For comparison, TLR4 and CD14 were analyzed in RAW cells, which were shown to respond to LPS (Figure 14C). TLR4 was expressed at a low level in RAW cells, but the difference is that its expression, as well as that of CD14, was significantly stimulated by LPS in RAW cells but not in D3 cells and D3-FBs (Figure 18B). These results explain the unresponsiveness of D3 cells and D3-FBs to LPS. The relative expressions of TLR4, CD14, and TNFR1 (the receptor that mediates the inflammatory response of TNFα) were also determined by RT-qPCR. The mRNA of the three molecules tested was expressed at the lowest levels in D3 cells, but it increased several fold in D3-FBs to levels comparable to 10T1/2 cells (Figure 18D, upper panel). It is interesting that the mRNA of TLR4 was not translated to functional TLR4 in D3-FBs. In the case of NFκB, its protein is readily detected in undifferentiated mESCs, as indicated by immunofluorescence microscopy. This was confirmed by flow cytometry. The expression of NFκB was further upregulated in D3-FBs (Figure 18C, indicated by the increased fluorescence intensity of the profile, D3 versus D3-FBs) to a level comparable to 10T1/2 cells. α-SMA as a marker of fibroblasts was extensively expressed in D3-FBs and 10T1/2 cells (Figure 18C). Therefore, the transition of NFκB from an inactive state in mESCs to an active state during differentiation is evident by its nuclear translocation and its increased expression.

It was noticed that the expression levels of TLR4 and TNFα receptors in D3-FBs increase slightly at higher passages, indicating that the signaling mechanisms undergo further development during subculturing. This is better demonstrated by the experiments in which cells were cultured for a prolonged period of time without splitting. As shown in Figure 18D (lower panel), the mRNA levels of TLR4, CD14, TNFR1, and NFκB all were progressively upregulated in the cells that were continuously cultured for 10 and 20 d. These cells showed increased induction of ICAM1 and IL-6 by TNFα, but they still lack a response to LPS (data not shown).

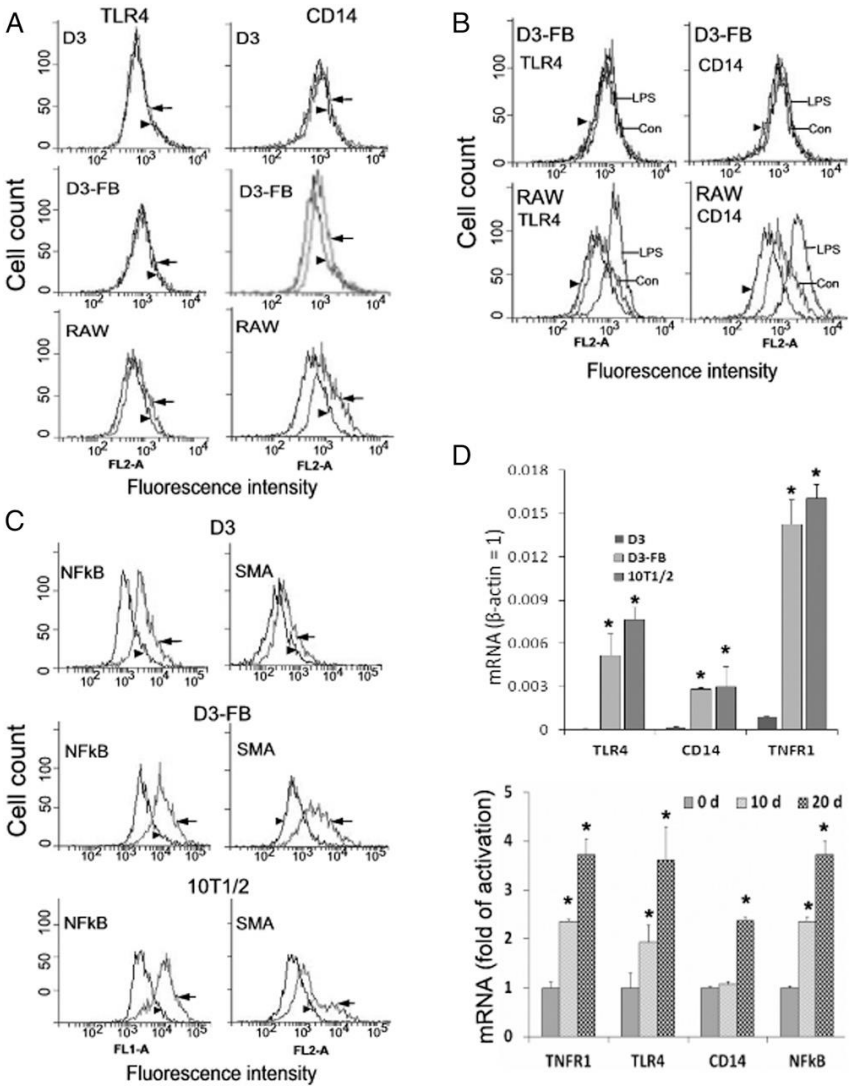

Figure 18. Differentiation-induced upregulation of signaling molecules that regulate inflammatory responses.

(A) Expression levels of TLR4 and CD14 in D3 cells, D3-FBs, and RAW cells determined by flow cytometry (the lines denoted by arrows). The lines denoted by arrowheads are negative controls. (B) The effect of LPS on the expression of TLR4 and CD14. The expression levels of TLR4 and CD14 in control cells and in cells that were treated with LPS for 20 h were determined by flow cytometry. The lines denoted by arrowheads represent negative controls. (C) Expression levels of NFκB and α-SMA in D3 cells, D3-FBs, and 10T1/2 cells determined by flow cytometry (denoted by arrows). The lines denoted by arrowheads are negative controls. (D) Relative mRNA levels of the indicated genes in D3 cells, D3-FBs, and 10T1/2 cells were determined by RT-qPCR (upper panel). The mRNA level of each gene was normalized to β-actin in each cell type. Long-term culture-induced upregulation of the indicated genes in D3-FBs was determined by RT-qPCR and compared with their mRNA levels in the cells before the long-term culture (0 d, designated as 1) (lower panel). Data are mean ± SD of representative experiments performed in triplicate that were repeated at least two times. *$p < 0.05$ versus D3 cells.

Discussion

The results described in this chapter extended our conclusion that ESCs have underdeveloped innate immunity, and were recently published in the Journal of Immunology (D'Angelo et al., 2017). A previous study demonstrated that mESCs were susceptible to bacterial infection, but they did not exhibit the immune and inflammatory responses typically displayed by somatic cells (Yu et al., 2009). This observation echoes the lack of IFN expression in virus- infected ESCs (Chen et al., 2010; Wang et al., 2014a). Because NFκB is a key transcription factor that mediates a broad spectrum of immune and inflammatory responses induced by numerous pathogens and cytokines (Baeuerle and Henkel, 1994), it was reasoned that the inactive status of NFκB in ESCs, as noted in virus-infected cells, could also account for the lack of immune and inflammatory responses in bacteria-infected ESCs. However, studies with LPS, which is widely used to replicate many aspects of bacterial infection, reported different results in ESCs. For example, Lee et al. (Lee et al., 2009) reported that LPS increased cell proliferation, with its receptor TLR4 mRNA positively detected in mESCs. In contrast, Taylor et al. (Taylor et al., 2010) showed that LPS inhibited mESC proliferation, but its conventional receptor TLR4 was not expressed. They proposed that the effect of LPS was mediated by TLR2 in the absence of TLR4 (Taylor et al., 2010). In contrast, the lack of response to LPS in mESCs and hESCs, and even their differentiated cells, was also reported (Földes et al., 2010; Zampetaki et al., 2006). The above-mentioned studies also disagreed on whether NFκB was activated by LPS. The only two studies that we are aware of that investigated the effect of TNFα also reported different results. One study suggests that mESCs and their differentiated vascular cells lack a response to TNFα (Zampetaki et al., 2007),

whereas the other indicates that TNFα negatively impacts mESC proliferation, viability, and differentiation (Wuu et al., 1998). Although the reasons for these discrepancies are not clear, the current study, based on the data obtained from mESCs and their differentiated cells via multiple experimental approaches, clearly demonstrates that mESCs have a deficient inflammatory response and provide the molecular basis for such deficiency in these cells.

Although best characterized as a key transcription factor in immune and inflammatory responses, NFκB also regulates a variety of cellular events (Hayden and Ghosh, 2012). Not surprisingly, the initial interest in NFκB in ESCs was its role in the regulation of their stem cell properties: pluripotency and differentiation. RelA and p50, the two subunits in the canonical NFκB pathway, are expressed at low levels in mESCs and hESCs, but they are upregulated upon differentiation (Armstrong et al., 2006; Kang et al., 2007; Kim et al., 2008; Torres and Watt, 2008). Furthermore, the activity of NFκB is repressed by Nanog, one of the key pluripotency genes in hESCs (Torres and Watt, 2008), and by miR-290 cluster in mESCs (Lüningschrör et al., 2012). These studies suggest that NFκB activity in ESCs is repressed as a mechanism to maintain pluripotency. Although none of the aforementioned studies investigated the role of NFκB in the context of innate immunity, their results support our conclusions. It is interesting that a recent study reported that hESCs were unable to respond to LPS, as described in mESCs in this study, but the NFκB pathway seemed to be functional (Földes et al., 2010), which is in contrast to another study showing that NFκB is not activated by TNFα in hESCs (Kang et al., 2007). The reasons for these different results are not clear, because the two studies used different hESC lines and assays. The data from hESCs in this study

clearly illustrate that they are similar to mESCs in the lack of NFκB activation and inflammatory response to LPS and TNFα. These results are also in accordance with the fact that mESCs and hESCs are deficient in the expression of type I IFN in the antiviral response, which is an NFκB-dependent process (Guo et al., 2015). Considering the highly conserved role of NFκB in innate immunity among different species of vertebrates (Baeuerle and Henkel, 1994), the findings in this study suggest that the lack of NFκB activation in response to immune and inflammatory stimuli is likely an intrinsic property of hESCs and mESCs.

In addition to the inactive status of NFκB, the absent or low-level expression of LPS and TNFα receptors seems to be another factor that contributes to the deficiency of the inflammatory response in mESCs. The differentiation process promoted the transition of NFκB to a functional status along with increased expression of NFκB. The results from experiments with TNFα provide a correlation among the expression levels of ICAM1 and IL-6, NFκB nuclear translocation, and the expression levels of TNFR1 in mESCs, mESC-DCs, and ESC-FBs. Zampetaki et al. (Zampetaki et al., 2007) reported that mESCs and even mESC-differentiated endothelial cells generated through EB differentiation were not responsive to TNFα. This result is similar to our observation in EB-differentiated endothelial cells. However, to the present data demonstrate that mESC-FBs are responsive to TNFα. Therefore, the inflammatory response mechanism is a developmentally regulated process that is affected by differentiation format, duration, and cell type. Although this study mainly used TNFα, the selected experiments performed with IL-1β showed similar results, indicating that the conclusions from TNFα could apply to other inflammatory cytokines whose actions depend on the activation of NFκB.

mESCs, mESC-DCs, and mESC-FBs all failed to show detectable responses to LPS, even in mESC-FBs that have a functional NFκB. This result can be explained by the lack of functional TLR4 expression in mESCs and mESC-FBs. It is intriguing that the mRNA of TLR4 was expressed, but it is not translated into functional proteins. The lack of LPS-induced gene expression is also noted in hESCs and their differentiated vascular cells (Földes et al., 2010). Studies showed that primary mouse embryonic fibroblasts are responsive to LPS (Kurt-Jones et al., 2004; Sacre et al., 2007). It is uncertain whether the lack of response of mESC-FBs to LPS is related to in vitro differentiation, because a similar observation was made in 10T1/2 cells. In addition to the results obtained from LPS and TNFα, the lack of an inflammatory response in mESCs was also demonstrated in response to viral infection, similar to the lack of IFN expression in CHIKV-infected mESCs (D'Angelo et al., 2016; Wang et al., 2014b).

Although the biological implications for the lack of an IFN-based antiviral response and an inflammatory response remain to be fully appreciated, it is not entirely surprising considering the fact that inflammatory cytokines and IFN are primarily produced for the purpose of defense and are negative regulators of growth and development (Hertzog et al., 1994). It is logical for ESCs not to produce these molecules at a developmental stage when cell proliferation and differentiation are major events, especially when ESCs reside in the womb where they could be protected by the mother's immune system (Levy, 2007). In this view, the innate immunity is not innate in ESCs but rather is developmentally acquired during differentiation by somatic cells, at least in cases of IFNβ-based antiviral and inflammatory mechanisms. Recent studies on this subject led to the notion that ESCs and differentiated somatic cells may have adapted

distinct defense mechanisms at different stages of organismal development (Guo, 2016; Pare and Sullivan, 2014).

Together with previous studies (D'Angelo et al., 2016; Földes et al., 2010; Glaser et al., 2011; Rajan et al., 2008; Sidney et al., 2014; Zampetaki et al., 2006, 2007), the data presented here demonstrate that commonly used in vitro differentiation methods only partially promote the development of innate immune response mechanisms. Questions remain as to how and to what degree the attenuated innate immunity may affect their therapeutic potential. To fully understand these questions, it will be essential to have a complete characterization of ESC-derived cells by in vitro and in vivo studies. Eventually, human cells will have to be used for therapeutic applications. However, like in many other areas of biomedical research, the knowledge derived from mouse models has been instrumental in understanding human physiology and diseases. The knowledge derived from studies of mESCs and mESC-DCs will be valuable for developing strategies to obtain clinically usable cells from human pluripotent stem cells.

CHAPTER V – CHARACTERIZATION OF EMBRYONIC STEM CELL-DERIVED FIBROBLASTS AS MESENCHYMAL STEM CELLS WITH ATTENUATED INNATE IMMUNITY

Introduction

During the analyses of innate immune development described in the preceding chapters, it was observed that when cultured for extended periods without passaging, mESC-FBs began to accumulate lipid droplets in the cytosol, reminiscent of adipocytes. Although these cells were originally characterized as fibroblasts, adipogenic differentiation is one characteristic of mesenchymal stem cells (MSCs), and thus I proceeded to examine mESC-FBs for other MSC characteristics.

While the field of stem-cell based regenerative medicine and tissue engineering has enormous potential in the treatment of injury and disease, the initial promise following the isolation and culture of human embryonic stem cells nearly two decades ago (Thomson et al., 1998) has not yet been fully realized. Although much progress has been made in defining proper differentiation conditions for desirable cell types, several barriers to widespread clinical use remain, such as the difficulty in obtaining sufficient yield and purity of desired cell types, potential immunogenicity resulting from allogeneic transplants, the risk of teratoma formation, and ethical and legal concerns. MSCs, on the other hand, are widely used in clinical settings and are currently being investigated in hundreds of clinical trials. MSCs are fibroblast-like cells first characterized by their capacity to differentiate into mesodermal cell types such as bone, cartilage, and fat cells in vitro (Shi et al., 2010). In addition to their differentiation capacity, MSCs produce large amounts of trophic and immunosuppressive factors and can “home” to sites of

injury to reduce inflammation and promote wound healing (Bianco et al., 2013). As inflammation is a key component in a multitude of diseases, the anti-inflammatory properties of MSCs make them potentially useful in myriad conditions, and indeed, the majority of ongoing clinical trials and for-profit stem cell clinics focus on these properties rather than the differentiation capacity of MSCs.

Originally isolated from bone marrow, cells with MSC characteristics have now been described from nearly every vascularized tissue, including readily available sources such as adipose tissue, dental pulp, and umbilical cord (Wada et al., 2013). Their restricted differentiation capacity and availability from adult tissues means that MSCs avoid many of the hurdles associated with clinical use of ESCs. However, isolation of MSCs often requires invasive harvesting procedures, they have limited in vitro expansion capacity, and donor variability can result in discrepancies in therapeutic value.

ESCs are a promising alternative source for MSC-like cells. While MSCs are rare in adult tissues and have poor expansion potential in vitro, ESCs have unlimited proliferative capacity, and their derived cells may retain unique properties that differ from their naturally-differentiated counterparts. It is demonstrated here that mESC-FBs display the major characteristics of MSCs, including morphology and marker expression, differentiation potential, and expression of immunosuppressive mediators and trophic factors. Additionally, the attenuated innate immune characteristics described in Chapters III and IV and expanded upon in this chapter represent a unique and possibly beneficial property not achievable with adult derived MSCs. Given its simplicity and short time course, our method of differentiation and isolation represents a significant improvement over existing methods.

Methods

Cell culture

D3 and DBA252 mESCs and mESC-FBs were cultured as described in the Methods section of Chapter III. Primary MSCs from mouse bone marrow (BM-MSCs) were obtained from an NIH funded research center at The Scripps Research Institute – Scripps Florida, Department of Molecular Therapeutics (Boregowda et al., 2016). RAW264.7 macrophages (ATCC) and BM-MSCs were cultured in 15% FBS DMEM and 100 U/mL penicillin and 100 mg/mL streptomycin and were maintained at 37°C in a humidified incubator with 5% CO_2.

Detection of tri-lineage differentiation by chemical staining

Cells were fixed with 4% paraformaldehyde for 15 minutes at 4°C, rinsed three times with PBS, and stained with 0.5% Oil Red O in isopropanol (for adipogenic staining), 2% Alizarin Red (for osteogenic staining), or 0.1% Safranin O (for chondrogenic stainin) for 10 minutes at RT, then rinsed and photographed under a phase contrast microscope.

Protein analysis by flow cytometry

Flow cytometry was carried out as described in the Methods section of Chapter III. Antibodies used in this study were purchased from BD Biosciences (CD105, CD44, and CD29) or Santa Cruz Biotechnology (CD34, CD45, COX2, iNOS).

mESC differentiation, reverse transcription real-time quantitative polymerase chain reaction (RT-qPCR), cell viability, immunocytochemistry and microscopic analysis, and statistical analysis were described in the Methods section of Chapter III.

Results

mESC-FBs display MSC morphology and marker expression

mESC-FBs were derived from in vitro differentiation of mESCs as described in the Methods section of Chapter III. Microscopic analysis shows that mESC-FBs display a flattened, spindle-shaped morphology similar to BM-MSCs (Figure 19, images). No definitive marker has been found to reliably distinguish MSCs. Instead, they are characterized by the lack of hematopoietic and endothelial markers and positive expression of a somewhat variable panel of surface markers (Dominici et al., 2006). It was found that mESC-FBs express mRNA of commonly reported positive MSC markers at similar levels to BM-MSCs, including CD106, CD105, CD73, CD29, and CD44, and do not express the hematopoietic/endothelial markers CD34 or CD31 (Figure 19A). Flow cytometry was used to analyze the expression of several markers at the protein level, which confirmed that mESC-FBs express cell surface CD105, CD29, and CD44, and lack expression of CD34 and CD45 (Figure 19B). Thus, mESC-FBs share similar morphology and marker expression with BM-MSCs.

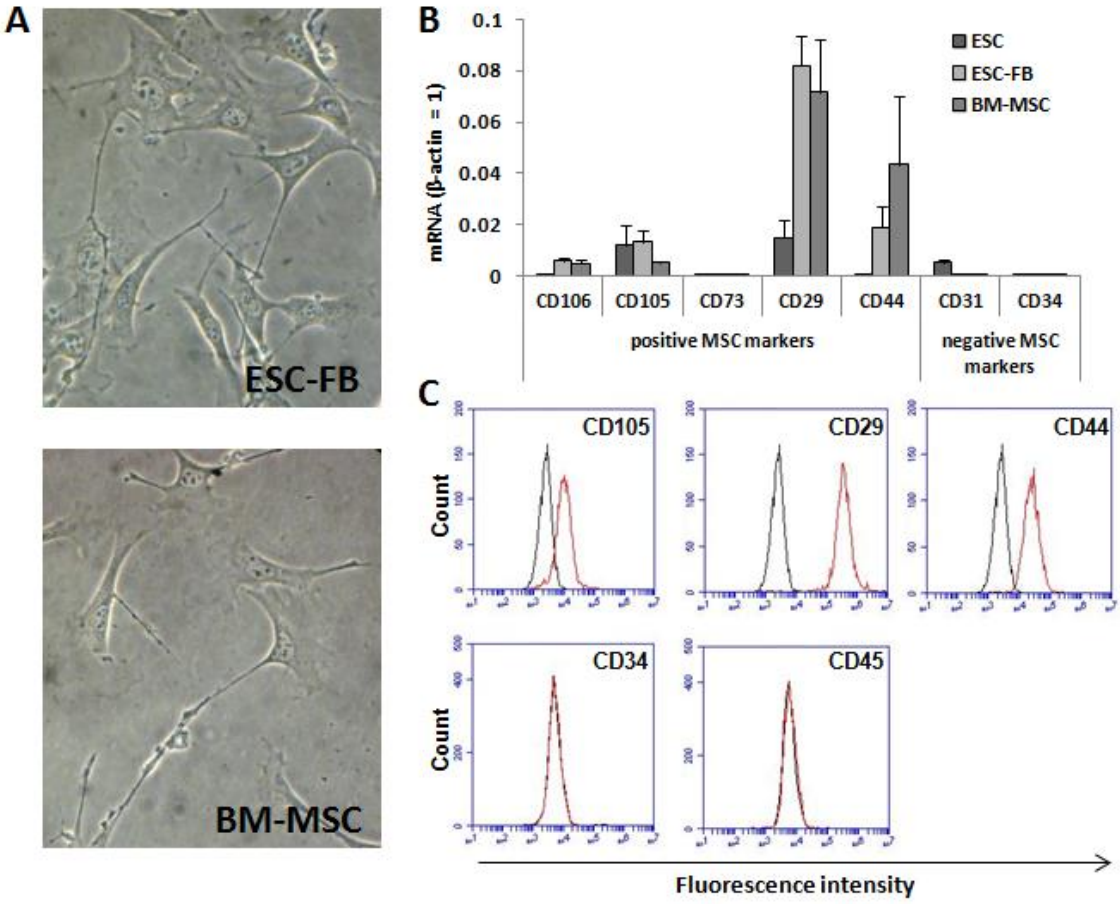

Figure 19. Morphology and MSC marker expression of mESC-FBs and BM-MSCs.

(A) mESC-FBs and BM-MSCs were photographed at 400x magnification under a phase contrast microscope in order to compare morphology. (B) Expression of commonly reported positive and negative markers for MSCs were analyzed by RT-qPCR in D3 ESCs, mESC-FBs from passages 3-6, and BM-MSCs at passage 2. Results are mean ± SD of three independent experiments. (C) Selected positive and negative MSC markers were measured in mESC-FBs by flow cytometry. The red curves represent cells stained with PE-conjugated antibodies specific for their respective targets, while the black curves represent cells stained with isotype control antibodies. Results are representative of three independent experiments.

mESC-FBs display tri-lineage differentiation potential characteristic of MSCs

MSCs characteristically display the capacity for osteogenic, chondrogenic, and adipogenic differentiation in vitro. In order to test the differentiation potential of mESC-FBs, the cells were grown to confluence and cultured in normal medium for up to 21 days without passaging. After about ten days, the cells began to accumulate multiple small

droplets in the cytosol and adopt a morphology reminiscent of brown adipocytes. Positive staining with Oil Red O dye (Reger et al., 2008) confirmed the presence of lipids in these intracytosolic vacuoles (Figure 20, bottom image), and RT-qPCR showed significant upregulation of the adipogenic differentiation marker C/EBPα after three weeks of differentiation (Figure 20, graph). Similarly, cultures stained positively with Alizarin Red, indicative of calcium deposition, and with Safranin O, which stains cartilage (Figure 20, top and middle images, respectively) (Boregowda et al., 2016). Additionally, three week cultures displayed significantly higher expression of the osteogenic differentiation marker OCN and the chondrocyte marker Sox9 (Figure 20, graph) compared with control cultures. These results indicate that mESC-FBs display the trilineage differentiation potential characteristic of MSCs.

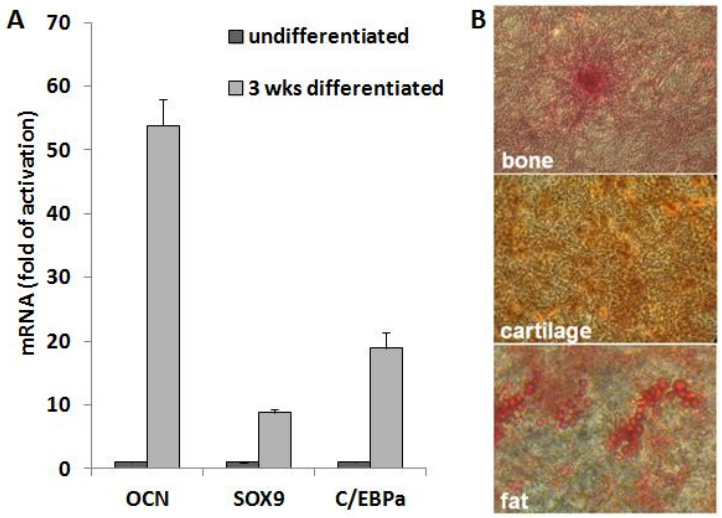

Figure 20. Tri-lineage differentiation capacity of mESC-FBs.

mESC-FBs were allowed to spontaneously differentiate by culturing in normal medium without passaging for 21 days. (A) Cells were then analyzed by RT-qPCR for expression of differentiation markers for osteogenisis (OCN), chondrogenesis (SOX9), and adipogenesis (C/EBPα). Values are mean ± SD of an experiment performed in triplicate and repeated three times. (B) Cells were fixed and stained for the products of differentiation with alizarin red (bone), safranin O (cartilage), or Oil Red O (fat). Images are representative of an experiment performed three times with similar results.

mESC-FBs express multiple trophic factors

MSCs produce high levels of multiple growth factors and other paracrine signaling molecules which can promote angiogenesis, cell survival, and wound healing (Ma et al., 2014). RT-qPCR analysis of several growth factors showed generally high basal level expression in mESC-FBs, similar to BM-MSCs used for comparison (Figure 21). Vascular-endothelial growth factor (VEGF) and stem cell factor (SCF) were expressed at similar levels in both cell types, while platelet-derived growth factor-B (PDGF-B) and hepatocyte growth factor (HGF) levels were significantly higher in mESC-FBs, and basic fibroblast growth factor (bFGF) and connective tissue growth factor (CTGF) were significantly higher in BM-MSCs. Interestingly, expression of stromal cell-derived factor-1 (SDF-1) was also significantly higher in mESC-FB (Figure 21, right panel). SDF-1, also known as CXCL12, is a chemokine with diverse functions. It has been implicated in the homing of MSCs to sites of inflammation in vivo, and is also chemotactic for immune cells and endothelial progenitors (Takano et al., 2014). In addition to stimulating migration of MSCs and other cell types, SDF-1 was also shown to increase survival of cardiac myocytes (Zhang et al., 2007).

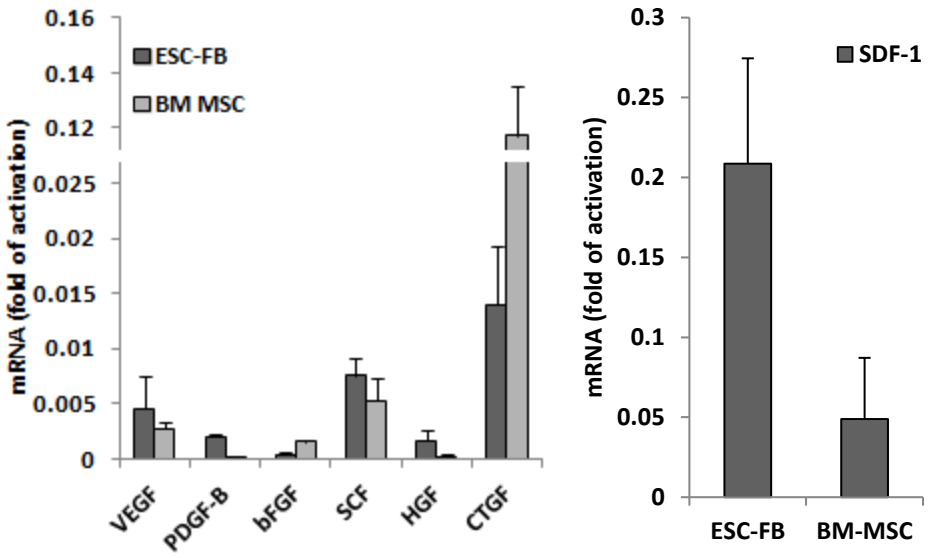

Figure 21. Basal expression of trophic factors in mESC-FBs.

Untreated mESC-FBs were analyzed by RT-qPCR for expression of angiogenic and trophic growth factors. Untreated BM-MSCs were tested for comparison. Values represent mean ± SD of samples from three independent experiments.

mESC-FBs express immunosuppressive mediators

Arguably the most attractive quality of MSCs for clinical use is their ability to potently suppress or reverse activation of immune cells to inhibit inflammation. This immunosuppression is achieved through a variety of mechanisms, many of which are mediated by locally acting molecules secreted by MSCs (Bernardo and Fibbe, 2013). Analysis of basal expression of several putative anti-inflammatory mediators shows mESC-FBs constitutively express COX-2, HO-1, and TGF-β, although their mRNA levels are somewhat lower than BM-MSCs (Figure 22A). Additionally, exposure to inflammatory cytokines has been shown to activate or enhance the anti-inflammatory actions of MSCs. Expression of these mediators was examined in response to treatment with TNFα, IL-1β, IFNγ, or combinations of these cytokines. COX2 and HO-1 mRNA levels remained high but relatively unchanged, but iNOS expression was significantly

induced under these conditions, especially by the combination of TNFα and IFNγ, cytokines known to act synergistically on expression of many genes (Ren et al., 2008). Examination of iNOS and COX2 protein by flow cytometry confirmed their significant upregulation under these conditions (Figure 22C). Notably, the expression of IL-10 was not detected before or after cytokine treatment in mESC-FBs (Figure 22, A and B). This cytokine has been reported to be involved in MSC-mediated suppression of immune cells, but whether it is expressed by MSCs or instead is induced in other cell types by MSCs has remained controversial (Prockop and Youn Oh, 2012). The results of the current study suggest the latter possibility, as IL-10 mRNA was also undetectable in BM-MSCs under our conditions (Figure 22A and data not shown).

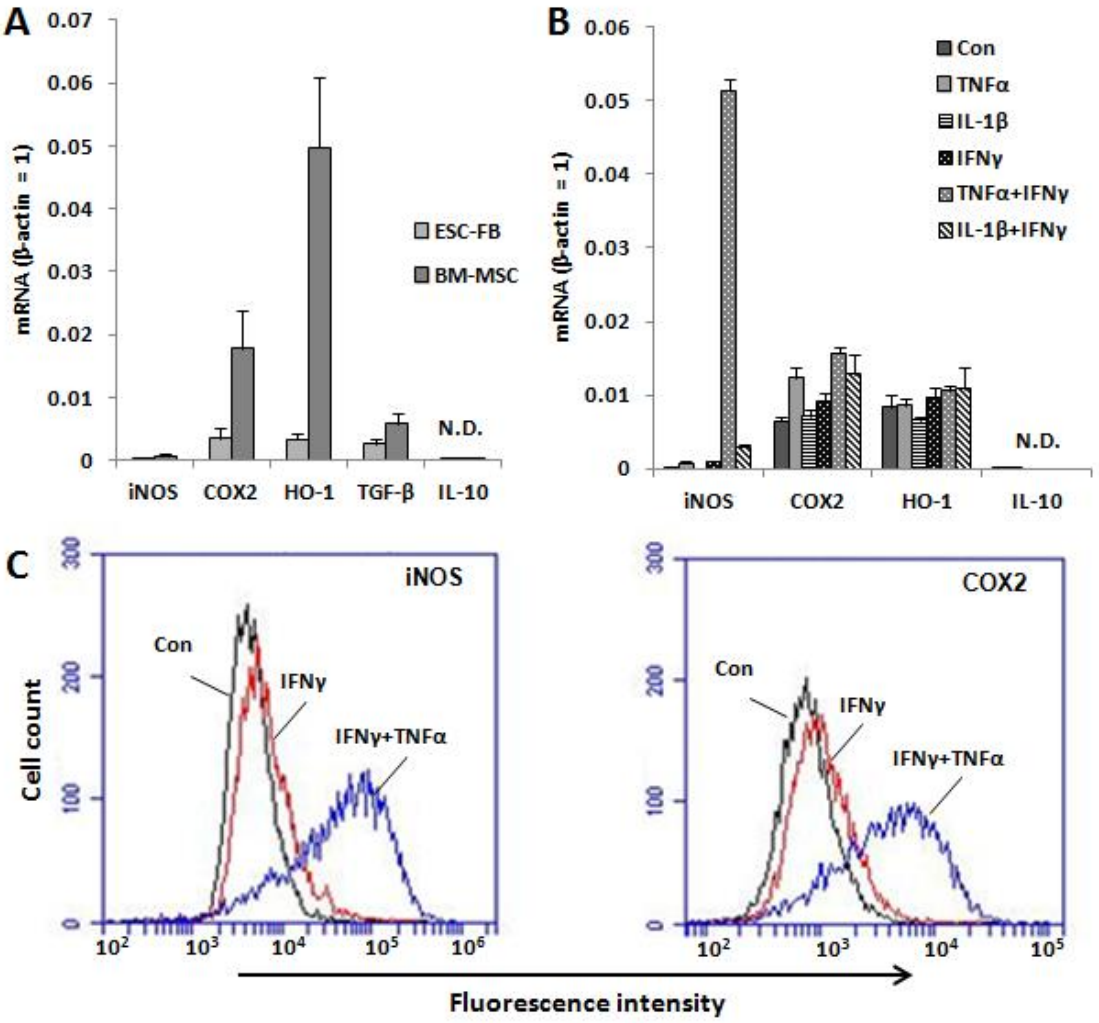

Figure 22. Expression of immunosuppressive mediators and their response to inflammatory cytokines.

(A) Basal levels of immunosuppressive genes were compared between mESC-FBs and BM-MSCs by RT-qPCR. Values are mean ± SD of samples from three independent experiments. (B) mESC-FBs were treated for 24 h with 20 ng/mL of the indicated inflammatory cytokines, alone or in combination, before qPCR analysis of immunosuppressive genes. Values are mean ± SD of a representative experiment performed in triplicate and repeated three times. (C) Protein levels of iNOS and COX2 were analyzed in mESC-FBs by flow cytometry. Black curves indicate untreated mESC-FBs, red curves indicate 24 h IFNγ-treated cells, and blue curves indicate cells treated with a combination of IFNγ and TNFα for 24 h. Data are from a representative experiment performed three times with similar results.

Effect of mESC-FBs on macrophage activation

In order to test the interaction of mESC-FBs with immune cells, the cells were grown in a transwell co-culture with RAW264.7 mouse macrophages. Macrophages are tissue-resident immune cells and are integral players in all stages of the inflammatory response in vivo (Chung and Son, 2014). In response to different immune stimuli, macrophages are polarized to distinct phenotypes that have been broadly characterized as pro-inflammatory and anti-inflammatory, referred to as M1 and M2, respectively. When exposed to bacterial stimuli such as LPS, macrophages adopt the M1 phenotype, characterized by high expression of inflammatory cytokines such as TNFα and production of radical oxygen and nitrogen species through enzymes such as iNOS. M2 macrophages are associated with resolution of inflammation, and thus have lower levels of TNFα and iNOS, and instead produce IL-10 and Arg-1 (Rath et al., 2014).

MSCs have been reported to suppress or reverse M1 polarization and promote the M2 phenotype (Cho et al., 2014; Gao et al., 2014; Maggini et al., 2010). In our system, treatment of RAW264.7 macrophages with LPS for 6 h induced significant upregulation of the M1 markers TNFα and iNOS. When macrophages were co-cultured overnight with BM-MSCs in transwell inserts, a decrease in the magnitude of induction of these markers was observed (Figure 23, A and B). However, we unexpectedly found an opposite result upon co-culture with mESC-FBs—induction of TNFα and iNOS in response to LPS was further increased in the presence of mESC-FBs (Figure 23, C and D). Furthermore, these markers were upregulated in macrophages co-cultured with mESC-FBs even in the absence of LPS treatment, an effect not seen during BM-MSC co-culture. Priming mESC-FBs or BM-MSCs before co-culture with TNFα and IFNγ, a combination that

upregulates several anti-inflammatory mediators in these cells, had no apparent effect on their interaction with the macrophages. No significant upregulation of M2 markers such as IL-10 or Arg-1 was observed under any conditions with either cell type (data not shown). Similar results were seen with longer times of co-culture and/or LPS treatment and when cells were grown in direct co-culture (i.e. without a transwell; data not shown). Thus, rather than inhibiting the inflammatory phenotype of macrophages, mESC-FBs seem to promote it under our conditions.

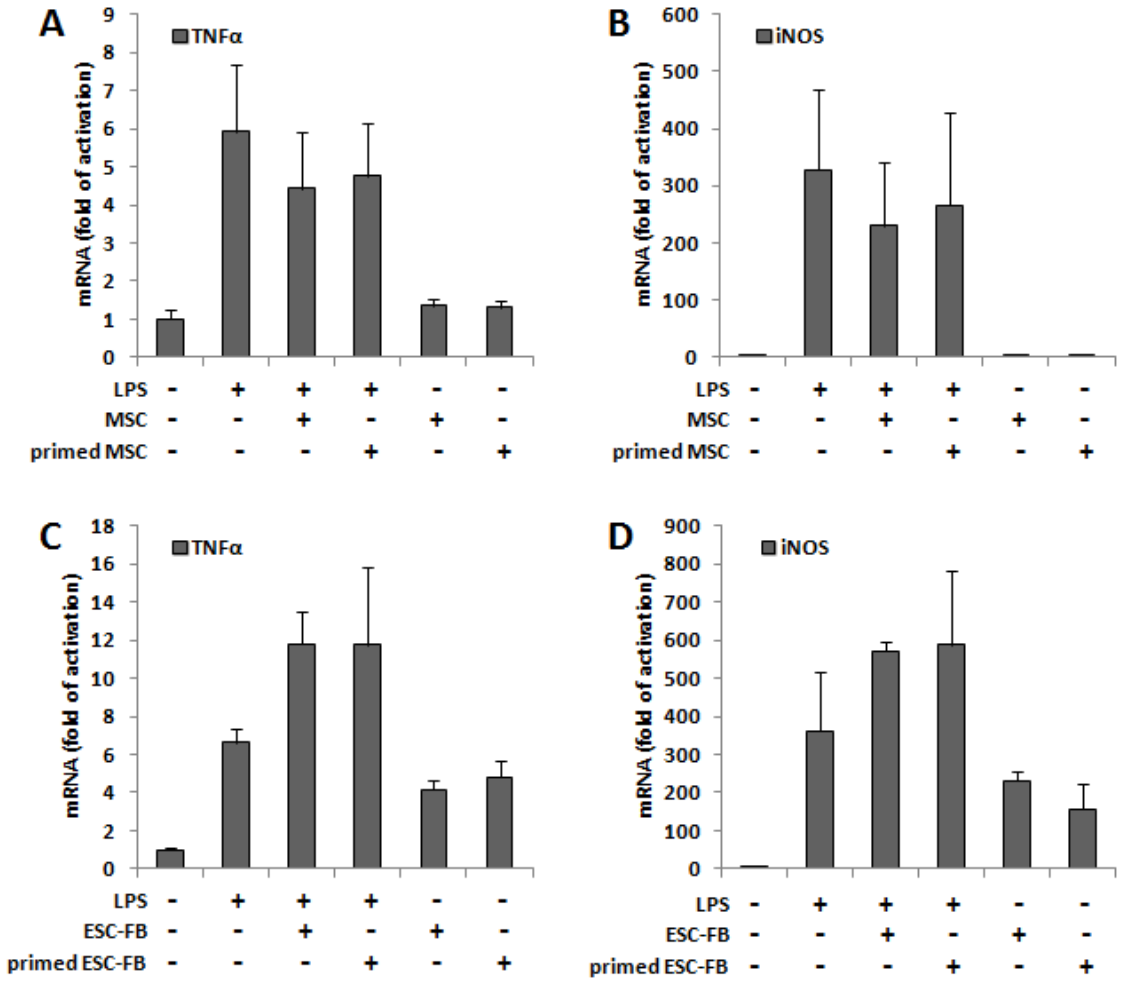

Figure 23. Effect of mESC-FBs on macrophage activation.

RAW264.7 macrophages were cultured alone or in overnight transwell co-culture with BM-MSCs (A and B) or mESC-FBs (C and D) before treatment with 1 ng/mL LPS for 6 h. Cells were then collected for RT-qPCR analysis of M1 and M2 markers. Values represent mean ± SD from three independent experiments.

mESC-FBs do not respond to LPS

During previous investigations of innate immune development during in vitro differentiation, mESC-FBs were used as a model to study the function of the NFκB pathway (D'Angelo et al., 2016, 2017). Pathway activation was tested by immunostaining for nuclear translocation of the p65 NFκB subunit in response to various stimuli, including polyIC, live La Crosse and chikungunya viruses, TNFα, IL-1α, and LPS. Surprisingly, it was found that LPS was unable to stimulate nuclear translocation, while all other stimuli tested induced robust activation (Figure 24A, bottom row; compare TNFα vs LPS). Multiple batches of LPS were tested at time points ranging from 30-120 minutes, but no nuclear staining was observed. In contrast, BM-MSCs showed robust nuclear translocation in response to both LPS and TNFα (Figure 24A, top row).

To confirm this lack of response by another method, RT-qPCR was used to test the expression levels of several marker genes downstream of LPS-induced signaling. While TNFα, IL-6, and ICAM-1 were all significantly induced in BM-MSCs after 6 h of LPS treatment, no significant upregulation was observed in mESC-FBs at any time point (Figure 24B).

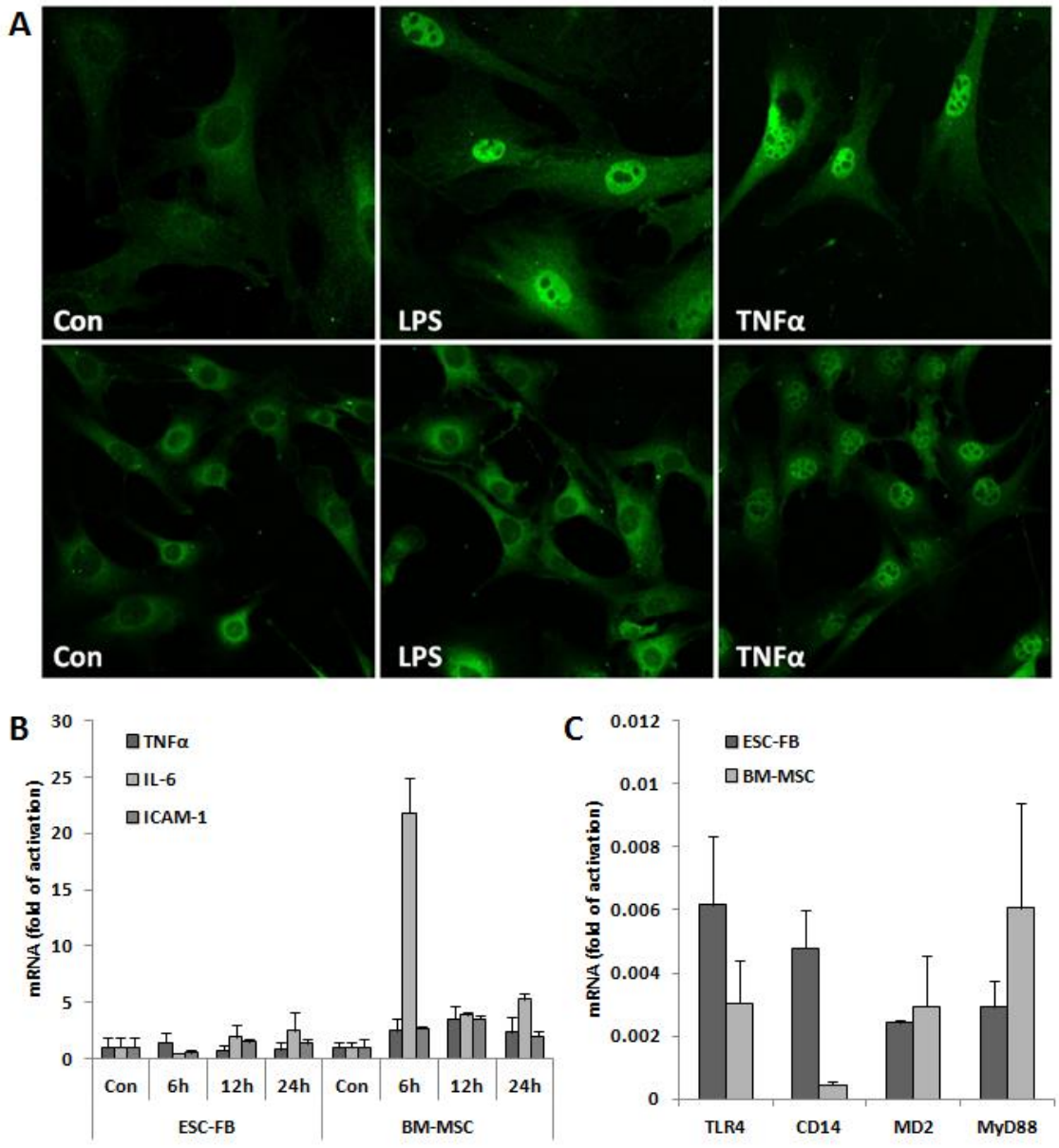

Figure 24. mESC-FBs do not respond to LPS.

(A) BM-MSCs (top row) and mESC-FBs (bottom row) were treated with 1 µg/mL LPS for 60 min, or with 20 ng/mL TNFα for 15 min. Cells were fixed with 3% paraformaldehyde and stained with a FITC-conjugated antibody specific for the p65 subunit of NFκB. The subcellular localization of NFκB was then assessed by confocal microscopy. Images are from a representative experiment performed three times with similar results. (B) Time course of expression of NFκB target genes in response to 1 µg/mL LPS in mESC-FBs and BM-MSCs. Results are mean ± SD of a representative experiment performed in triplicate and repeated three times. (C) Basal expression of LPS response pathway genes in mESC-FBs and BM-MSCs. Values are mean ± SD of samples from three independent experiments.

In order to further probe the reasons behind the lack of LPS responsiveness, the expression levels of several LPS response pathway components were examined in mESC-FBs. LPS-induced signaling occurs through a receptor complex composed of TLR4 together with its co-receptors CD14 and MD-2, as well as LPS-binding protein (LBP), which is present in serum. The activated receptor complex then signals through several intermediates including MyD88, resulting eventually in NFκB activation (Kawai and Akira, 2010). RT-qPCR analysis showed that mESC-FBs express TLR4, CD14, MD-2, and MyD88 mRNA at levels similar to or even higher than BM-MSCs (Figure 24C). However, as shown in Figure 18A in the Results section of Chapter 4, TLR4 protein was undetectable by flow cytometry in mESC-FB, although CD14 was clearly expressed, thus providing an explanation for the lack of responsiveness to LPS, although the reason for the discrepancy between TLR4 mRNA and protein expression is still unclear.

To test for a possible functional consequence of this unresponsiveness, cell viability was compared between mESC-FBs and BM-MSCs grown for four days in the presence of LPS with added IFNγ. Neither LPS nor IFNγ alone caused detectable cell death in either cell type (data not shown), but their combination caused moderate toxicity in BM-MSCs (Figure 25). Thus, the lack of response of mESC-FBs to LPS could conceivably protect them from toxicity in the presence of bacterial pathogens. This could confer an important advantage in a transplantation setting, where cells are likely to encounter contaminating bacteria.

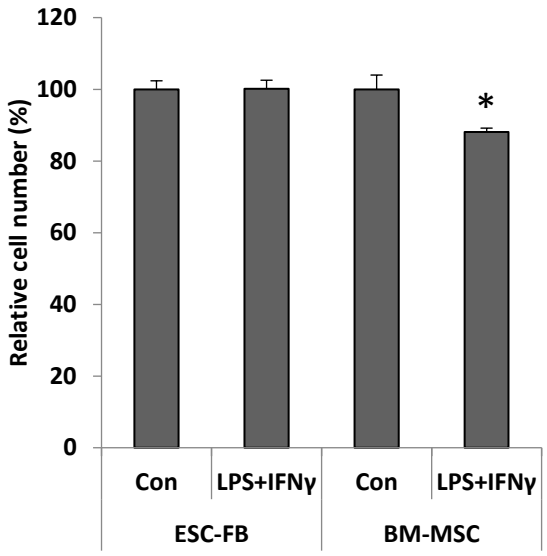

Figure 25. Comparison of mESC-FB and BM-MSC cell viability response to LPS and IFNγ.

mESC-FBs and BM-MSCs were treated with 1 μg/mL LPS and 20 ng/mL IFNγ for 4 days, then cells were stained with TB and optical density was measured as an indicator of cell number. Values are mean ± SD of a representative experiment performed in triplicate and repeated three times. *P < 0.05 compared with control cells.

Discussion

MSCs are at the forefront of clinical translation of stem cell-based regenerative medicine applications. However, their isolation requires invasive procedures and their poor expansion capacity makes it difficult to obtain a clinically useful number of cells. In this study, I have characterized mESC-derived fibroblasts according to the accepted criteria for MSCs, including plastic adherence, marker expression, and capacity for differentiation to bone, cartilage, and fat cells (Dominici et al., 2006). mESC-FBs express high levels of angiogenic and trophic growth factors and immunosuppressive mediators, similar to bone marrow-derived MSCs. Thus, ESCs can serve as an unlimited source for cells with the characteristics of MSCs.

Generation of MSCs from ESCs has been previously reported, but the method described here represents a substantial improvement. Most prior reports involve differentiation of ESCs in an EB format. Spontaneous differentiation of ESCs in EBs results in a mixed population consisting of a variety of lineages and differentiation states (Guo et al., 2007). Previous studies mainly rely on scraping cells at the periphery of EB outgrowths (the "raclure" method), or cell sorting using a flow cytometer or magnetic beads to isolate a population of cells based on one or two surface markers such as CD105 (Olivier et al., 2006). In our method, a ten-day treatment with retinoic acid is used as a potent driver of differentiation, and the resultant mESC-FBs are isolated based on their plastic adherence in culture, a property which has been used previously to purify primary fibroblasts and MSCs (Friedenstein et al., 1976; Owen, 1988). This results in a homogeneous population of cells with MSC characteristics and marker expression without the need for cell sorting methods, and in a time frame of less than two weeks.

In this study, I demonstrated the upregulation of marker genes for osteogenic, chondrogenic, and adipogenic lineages after prolonged culture without passaging (Figure 20). In addition, I demonstrated positive histochemical staining for the products of differentiation. These results demonstrate that mESC-FBs can spontaneously differentiate to characteristic mesodermal lineages in post-confluent cultures. As mESC-FBs are not a clonal population, it is unclear whether individual cells display this trilineage potential, or whether the population consists of different progenitors for each lineage. It is possible that some cells are already primed toward specific lineages, or that post-proliferative cells begin to differentiate in a stochastic manner or in response to paracrine factors released by other cells in the culture. Most reports on MSCs make use of specialized media with

added supplements to direct differentiation toward osteogenic, chondrogenic, or adipogenic lineages, although spontaneous differentiation has been reported before in adipose-derived MSCs (Dudakovic et al., 2014). It is likely that differentiation efficiency of mESC-FBs could be increased with the use of defined differentiation media, although that was not our focus in this study.

A major reason behind the clinical utility of MSCs is their high production of trophic and anti-inflammatory growth factors, cytokines, and other paracrine mediators (Caplan and Dennis, 2006). Comparison of mESC-FBs with BM-MSCs showed similarly high basal expression of multiple angiogenic and trophic growth factors (Figure 21), as well as constitutive expression of COX2 and HO-1 (Figure 22), two enzymes involved in the production of anti-inflammatory mediators (PGE2 and CO, respectively). Additionally, treatment with inflammatory cytokines, especially the combination of TNFα and IFNγ, resulted in strong induction of iNOS, the enzyme responsible for NO production (Figure 22). All of these mechanisms have been implicated in MSC-mediated suppression of inflammation, and their induction under inflammatory conditions is characteristic of MSCs (English, 2013).

Because the expression profile of immunosuppressive mediators in mESC-FBs matches that described for MSCs, the results obtained from macrophage co-culture experiments were unexpected. Numerous reports demonstrate that MSCs can reverse M1 polarization and/or promote M2 polarization in macrophages (Cho et al., 2014; Maggini et al., 2010). In our system, it was found that MSC co-culture modestly decreased expression of TNFα and iNOS in LPS-treated macrophages (Figure 23, A and B), while co-culture with mESC-FBs increased their expression (Figure 23, C and D). Additionally,

these markers were increased in macrophages upon co-culture with mESC-FBs even in the absence of LPS treatment (Figure 23, C and D). Expression of the M2 markers IL-10 and Arg-1 was negligible under any condition as measured by RT-qPCR. The implications of these findings are not clear. While mESC-FBs seem to increase the inflammatory phenotype of macrophages, the true effect in an in vivo setting is difficult to extrapolate from a simple in vitro experiment. While iNOS expression in macrophages is associated with their inflammatory state and is important for pathogen killing (Rath et al., 2014), its product, NO, also acts as a signaling molecule and a potent suppressor of T-cell proliferation and activation (Sato et al., 2007). Thus, further experimentation is required to determine the net effect of mESC-FBs on acute tissue inflammation, especially in an in vivo setting.

The finding that mESC-FBs are unable to respond to LPS (Figure 24) is interesting. Previous work has shown that while mESCs have negligible responses to viral, bacterial, or inflammatory stimuli, their ability to respond to viral stimuli and inflammatory cytokines is acquired during in vitro differentiation (D'Angelo et al., 2016, 2017). But TLR4 protein expression in mESC-FBs was not detected (see Chapter IV, Figure 18), although its mRNA was expressed at high level (Figure 24C). This suggests that different parts of the innate immune system develop at different rates during in vitro differentiation. The mechanism behind the discrepancy between mRNA and protein levels is unclear, but suggests potential miRNA-based repression. Regardless of the mechanism, the present work showed that the attenuated innate immunity (i.e. lack of LPS response) could have functional advantages for mESC-FBs over BM-MSCs, as they did not produce inflammatory cytokines which could augment local inflammation in a

transplantation setting (Figure 24B), and they were not susceptible to LPS-induced cell death, as were BM-MSCs (Figure 25).

In summary, this study demonstrated that mESC-FBs display the major characteristics of MSCs, including morphology, marker expression, and differentiation capacity. These cells express high levels of trophic growth factors, and express immunosuppressive mediators both constitutively and when induced by inflammatory cytokines. They can alter the phenotype of macrophages in in vitro co-culture, although further study is needed to accurately characterize their net effect as pro- or anti-inflammatory. And mESC-FBs derived by our method do not respond to LPS, which was shown to be a potential advantage in a transplantation setting where contact with bacteria is likely. Taking into account these characteristics, our derivation method warrants study in human cells, where ESCs could serve as a promising and unlimited source of clinically useful MSCs.

CHAPTER VI – CONCLUSION

In these three related projects, the major findings are that innate immunity can be partially acquired during in vitro differentiation of ESC, and this development can be modulated through manipulation of culture conditions to obtain cells with unique characteristics compared with naturally-differentiated counterparts. I first demonstrated that innate antiviral immunity, which is absent in mESCs, can be acquired during in vitro differentiation. Two different methods of differentiation were used: EB formation, resulting in a mixed population of differentiated cells of multiple lineages; and RA-induced differentiation of fibroblast-like cells (mESC-FBs) which were purified based on plastic adherence. Differentiation via either format resulted in cells with significantly increased responsiveness to dsRNA transfection or infection with live viruses, as measured by induction of the IFNβ gene. This differentiation coincided with an increase in the expression of PRRs that detect dsRNA (i.e. TLR3, RIG-I, and PKR). The magnitude of IFNβ induction in response to viral stimuli increased further with continued passaging of mESC-FBs, as did response to exogenously added IFN as measured by induction of ISGs. However, the magnitude of responses was still significantly lower than that of naturally-differentiated fibroblasts, even after 50 passages. Innate immune development could be significantly accelerated by priming the cells with low doses of polyIC during propagation. Importantly, it was demonstrated that NFκB is not activated by viral infection in mESCs, but becomes active in mESC-FBs.

In the second project, the lack of innate immune responses in mESCs was expanded to inflammatory stimuli. It was shown that mESCs are unresponsive to TNFα, IL-1β, or LPS, as assayed by NFκB translocation, IκB degradation, and target gene

expression. After differentiation through EB formation or RA treatment, NFκB became functional in response to inflammatory cytokines but not to LPS. Differentiation was also found to sensitize the cells to TNFα-induced cytotoxicity in the presence of the transcription inhibitor actinomycin D or in conjunction with viral infection. As was found with antiviral PRRs, the expression of NFκB and inflammatory cytokine receptors increased during differentiation, providing a mechanistic explanation of both the lack of innate immune responsiveness in mESCs and its acquisition during differentiation.

These studies provide insight into a question that is potentially crucial for the use of ESC-derived cells in regenerative medicine application and yet is often overlooked—whether ESCs and their derived cells have competent innate immunity. I showed conclusively that innate immunity is acquired during in vitro differentiation, but the magnitude of responsiveness lags behind that of naturally-differentiated cells, a deficiency which can be overcome with immune priming during culturing.

Finally, I characterized mESC-FBs according to the criteria used to identify MSCs. The ease of derivation and purification of MSC-like mESC-FBs represents an improvement over existing methods. mESC-FBs demonstrate morphology and marker expression similar to MSCs, as well as characteristic trilineage differentiation capacity to bone, cartilage, and fat cells. Additionally, the cells express high levels of trophic and immunosuppressive factors, a major trait that makes MSCs desirable for clinical use. mESC-FBs seem to promote an inflammatory phenotype in co-cultured macrophages, contrary to the reported effects of BM-MSCs, although more in depth study is required on the potential implications of this effect on overall tissue inflammation in an in vivo setting. Finally, mESC-FBs do not express TLR4 at the protein level and thus do not

respond to LPS, an as yet unexplained but potentially advantageous trait that could prevent the cells from contributing to inflammation and protect them from LPS-induced cell death in an infection setting. These results show that ESCs can serve as a promising and unlimited source of cells with MSC-like characteristics and with unique and potentially useful traits owing to their ESC source.

In summary, the above studies represent a substantial contribution to the field of stem cell research. The findings regarding the acquisition of innate immunity in vitro and the key role of the NFκB pathway not only offer insight into the developmental biology of innate immunity, but also have important implications for clinical use of ESC-derived cells. And the derivation of mESC-FBs as MSCs constitutes an improved method for obtaining therapeutically useful cells, a method that warrants further study in human cells.

REFERENCES

Acharya, D., Paul, A.M., Anderson, J.F., Huang, F., and Bai, F. (2015). Loss of Glycosaminoglycan Receptor Binding after Mosquito Cell Passage Reduces Chikungunya Virus Infectivity. PLoS Negl. Trop. Dis. *9*, e0004139.

De Almeida, P.E., Ransohoff, J.D., Nahid, A., and Wu, J.C. (2013). Immunogenicity of pluripotent stem cells and their derivatives. Circ. Res. 112, 549–561.

Armstrong, L., Hughes, O., Yung, S., Hyslop, L., Stewart, R., Wappler, I., Peters, H., Walter, T., Stojkovic, P., Evans, J., et al. (2006). The role of PI3K/AKT, MAPK/ERK and NFkappabeta signalling in the maintenance of human embryonic stem cell pluripotency and viability highlighted by transcriptional profiling and functional analysis. Hum. Mol. Genet. 15, 1894–1913.

Baeuerle, P., and Henkel, T. (1994). Function and Activation of NF-kappaB in the Immune System. Annu. Rev. Immunol. 12, 141–179.

Bai, F., Wang, T., Pal, U., Bao, F., Gould, L.H., and Fikrig, E. (2005). Use of RNA interference to prevent lethal murine West Nile virus infection. J. Infect. Dis. 191, 1148–1154.

Bell, E. (2009). Innate immunity: Endothelial cells as sentinels. Nat. Rev. Immunol. 9, 532–533.

Bernardo, M.E., and Fibbe, W.E. (2013). Mesenchymal stromal cells: sensors and switchers of inflammation. Cell Stem Cell 13, 392–402.

Bianco, P., Cao, X., Frenette, P.S., Mao, J.J., Robey, P.G., Simmons, P.J., and Wang, C.-Y. (2013). The meaning, the sense and the significance: translating the science of mesenchymal stem cells into medicine. Nat. Med. 19, 35–42.

Blancas, A.A., Lauer, N.E., and McCloskey, K.E. (2008). Endothelial differentiation of embryonic stem cells. In Current Protocols in Stem Cell Biology, (Hoboken, NJ, USA: John Wiley & Sons, Inc.), p. Unit 1F.5.

Boregowda, S. V, Krishnappa, V., and Phinney, D.G. (2016). Isolation of Mouse Bone Marrow Mesenchymal Stem Cells. In Methods in Molecular Biology (Clifton, N.J.), pp. 205–223.

Boyd, A.S., and Wood, K.J. (2009). Variation in MHC expression between undifferentiated mouse ES cells and ES cell-derived insulin-producing cell clusters. Transplantation 87, 1300–1304.

Boyer, L. a., Lee, T.I., Cole, M.F., Johnstone, S.E., Stuart, S., Zucker, J.P., Guenther, M.G., Kumar, R.M., Murray, H.L., Jenner, R.G., et al. (2005). Core Transcriptional Regulatory Circuitry in Human Embryonic Stem Cells. Cell 122, 947–956.

Brook, F. a, and Gardner, R.L. (1997). The origin and efficient derivation of embryonic stem cells in the mouse. Proc. Natl. Acad. Sci. U. S. A. 94, 5709–5712.

Burke, D.C., Graham, C.F., and Lehman, J.M. (1978). Appearance of interferon inducibility and sensitivity during differentiation of murine teratocarcinoma cells in vitro. Cell 13, 243–248.

Caplan, A.I. (2008). All MSCs Are Pericytes? Cell Stem Cell 3, 229–230.

Caplan, A.I., and Dennis, J.E. (2006). Mesenchymal stem cells as trophic mediators. J. Cell. Biochem. 98, 1076–1084.

Chen, L., and Daley, G.Q. (2008). Molecular basis of pluripotency. Hum. Mol. Genet. 17.

Chen, G.-Y., Hwang, S.-M., Su, H.-J., Kuo, C.-Y., Luo, W.-Y., Lo, K.-W., Huang, C.-C., Chen, C.-L., Yu, S.-H., and Hu, Y.-C. (2012). Defective antiviral responses of induced pluripotent stem cells to baculoviral vector transduction. J. Virol. 86, 8041–8049.

Chen, L.-L., Yang, L., and Carmichael, G.G. (2010). Molecular basis for an attenuated cytoplasmic dsRNA response in human embryonic stem cells. Cell Cycle 9, 3552–3564.

Cho, D.-I., Kim, M.R., Jeong, H., Jeong, H.C., Jeong, M.H., Yoon, S.H., Kim, Y.S., and Ahn, Y. (2014). Mesenchymal stem cells reciprocally regulate the M1/M2 balance in mouse bone marrow-derived macrophages. Exp. Mol. Med. 46, e70.

Chung, E., and Son, Y. (2014). Crosstalk between mesenchymal stem cells and macrophages in tissue repair. Tissue Eng. Regen. Med. 11, 431–438.

Covas, D.T., Panepucci, R.A., Fontes, A.M., Silva, W.A., Orellana, M.D., Freitas, M.C.C., Neder, L., Santos, A.R.D., Peres, L.C., Jamur, M.C., et al. (2008). Multipotent mesenchymal stromal cells obtained from diverse human tissues share functional properties and gene-expression profile with CD146+ perivascular cells and fibroblasts. Exp. Hematol. 36, 642–654.

Crisan, M., Yap, S., Casteilla, L., Chen, C.W., Corselli, M., Park, T.S., Andriolo, G., Sun, B., Zheng, B., Zhang, L., et al. (2008). A Perivascular Origin for Mesenchymal Stem Cells in Multiple Human Organs. Cell Stem Cell 3, 301–313.

Cullen, B.R., Cherry, S., and Tenoever, B.R. (2013). Is RNA interference a physiologically relevant innate antiviral immune response in mammals? Cell Host Microbe 14, 374–378.

D'Angelo, W., Acharya, D., Wang, R., Wang, J., Gurung, C., Chen, B., Bai, F., and Guo, Y.-L. (2016). Development of Antiviral Innate Immunity During In Vitro Differentiation of Mouse Embryonic Stem Cells. Stem Cells Dev. 25, 648–659.

D'Angelo, W., Gurung, C., Acharya, D., Chen, B., Ortolano, N., Bai, F., and Guo, Y.-L. (2017). The Molecular Basis for the Lack of Inflammatory Responses in Mouse Embryonic Stem Cells and Their Differentiated Cells. J. Immunol. 198, 1601068.

Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F., Krause, D., Deans, R., Keating, A., Prockop, D., and Horwitz, E. (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8, 315–317.

Drukker, M., Katz, G., Urbach, A., Schuldiner, M., Markel, G., Itskovitz-Eldor, J., Reubinoff, B., Mandelboim, O., and Benvenisty, N. (2002). Characterization of the expression of MHC proteins in human embryonic stem cells. Proc. Natl. Acad. Sci. U. S. A. 99, 9864–9869.

Dudakovic, A., Camilleri, E., Riester, S.M., Lewallen, E.A., Kvasha, S., Chen, X., Radel, D.J., Anderson, J.M., Nair, A.A., Evans, J.M., et al. (2014). High-resolution molecular validation of self-renewal and spontaneous differentiation in clinical-grade adipose-tissue derived human mesenchymal stem cells. J. Cell. Biochem. 115, 1816–1828.

English, K. (2013). Mechanisms of mesenchymal stromal cell immunomodulation. Immunol. Cell Biol. 91, 19–26.

English, K., and Wood, K.J. (2011). Immunogenicity of embryonic stem cell-derived progenitors after transplantation. Curr. Opin. Organ Transplant. 16, 90–95.

Enzerink, A., and Vaheri, A. (2011). Fibroblast activation in vascular inflammation. J. Thromb. Haemost. 9, 619–626.

Földes, G., Liu, A., Badiger, R., Paul-Clark, M., Moreno, L., Lendvai, Z., Wright, J.S., Ali, N.N., Harding, S.E., and Mitchell, J.A. (2010). Innate immunity in human embryonic stem cells: comparison with adult human endothelial cells. PLoS One 5, e10501.

Friedenstein, A.J., Gorskaja, J.F., and Kulagina, N.N. (1976). Fibroblast precursors in normal and irradiated mouse hematopoietic organs. Exp. Hematol. 4, 267–274.

Gao, S., Mao, F., Zhang, B., Zhang, L., Zhang, X., Wang, M., Yan, Y., Yang, T., Zhang, J., Zhu, W., et al. (2014). Mouse bone marrow-derived mesenchymal stem cells induce macrophage M2 polarization through the nuclear factor-κB and signal transducer and activator of transcription 3 pathways. Exp. Biol. Med. (Maywood). 239, 366–375.

García, M.A., Meurs, E.F., and Esteban, M. (2007). The dsRNA protein kinase PKR: Virus and cell control. Biochimie 89, 799–811.

Geijtenbeek, T.B.H., and Gringhuis, S.I. (2009). Signalling through C-type lectin receptors: shaping immune responses. Nat. Rev. Immunol. 9, 465–479.

Gil, J., and Esteban, M. (2000). Induction of apoptosis by the dsRNA-dependent protein kinase (PKR): mechanism of action. Apoptosis 5, 107–114.

Glaser, D.E., Gower, R.M., Lauer, N.E., Tam, K., Blancas, A.A., Shih, A.J., Simon, S.I., and McCloskey, K.E. (2011). Functional characterization of embryonic stem cell-derived endothelial cells. J. Vasc. Res. 48, 415–428.

González-Navajas, J.M., Lee, J., David, M., and Raz, E. (2012). Immunomodulatory functions of type I interferons TL - 12. Nat. Rev. Immunol. 12 VN-r, 125–135.

Gronthos, S., Mankani, M., Brahim, J., Robey, P.G., and Shi, S. (2000). Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proc. Natl. Acad. Sci. USA 97, 13625–13630.

Guo, Y.-L. (2016). Utilization of different anti-viral mechanisms by mammalian embryonic stem cells and differentiated cells. Immunol. Cell Biol. 1–29.

Guo, Y.-L., Ye, J., and Huang, F. (2007). p38alpha MAP kinase-deficient mouse embryonic stem cells can differentiate to endothelial cells, smooth muscle cells, and neurons. Dev. Dyn. 236, 3383–3392.

Guo, Y.-L., Chakraborty, S., Rajan, S.S., Wang, R., and Huang, F. (2010). Effects of oxidative stress on mouse embryonic stem cell proliferation, apoptosis, senescence, and self-renewal. Stem Cells Dev. 19, 1321–1331.

Guo, Y.-L., Carmichael, G.G., Wang, R., Hong, X., Acharya, D., Huang, F., and Bai, F. (2015). Attenuated Innate Immunity in Embryonic Stem Cells and Its Implications in Developmental Biology and Regenerative Medicine. Stem Cells 33, 3165–3173.

Guo, Y.L., Baysal, K., Kang, B., Yang, L.J., and Williamson, J.R. (1998). Correlation between sustained c-Jun N-terminal protein kinase activation and apoptosis induced by tumor necrosis factor-alpha in rat mesangial cells. J. Biol. Chem. 273, 4027–4034.

Guo, Y.L., Kang, B., Yang, L.J., and Williamson, J.R. (1999). Tumor necrosis factor-alpha and ceramide induce cell death through different mechanisms in rat mesangial cells. Am. J. Physiol. 276, F390-397.

Haniffa, M.A., Wang, X., Holtick, U., Rae, M., Isaacs, J.D., Dickinson, A.M., Hilkens, C.M.U., and Collin, M.P. (2007). Adult human fibroblasts are potent immunoregulatory cells and functionally equivalent to mesenchymal stem cells. J. Immunol. 179, 1595–1604.

Haniffa, M.A., Collin, M.P., Buckley, C.D., and Dazzi, F. (2009). Mesenchymal stem cells: the fibroblasts' new clothes? Haematologica 94, 258–263.

Harada, H., Willison, K., Sakakibara, J., Miyamoto, M., Fujita, T., and Taniguchi, T. (1990). Absence of the type I IFN system in EC cells: Transcriptional activator (IRF-1) and repressor (IRF-2) genes are developmentally regulated. Cell 63, 303–312.

Hayden, M.S., and Ghosh, S. (2012). NF-κB, the first quarter-century: remarkable progress and outstanding questions. Genes Dev. 26, 203–234.

Hayden, M.S., West, a P., and Ghosh, S. (2006). NF-kappaB and the immune response. Oncogene 25, 6758–6780.

Hedrick, M.H., Zuk, P.A., Zhu, M., Ashjian, P., De Ugarte, D.A., Huang, J.I., Mizuno, H., Alfonso, Z.C., Fraser, J.K., and Benhaim, P. (2002). Human Adipose Tissue Is a Source of Multipotent Stem Cells. Mol. Biol. Cell 13, 4279–4295.

Hematti, P. (2012). Mesenchymal stromal cells and fibroblasts: a case of mistaken identity? Cytotherapy 14, 516–521.

Hertzog, P.J., Hwang, S.Y., and Kola, I. (1994). Role of interferons in the regulation of cell proliferation, differentiation, and development. Mol. Reprod. Dev. 39, 226–232.

Huang, C.C., Duffy, K.E., Mateo, L.R.S., Amegadzie, B.Y., Sarisky, R.T., and Mbow, M.L. (2006). A pathway analysis of poly (I:C)-induced global gene expression change in human peripheral blood mononuclear cells. Physiol. Genomics 26, 125–133.

Jordana, M., Särnstrand, B., Sime, P.J., and Ramis, I. (1994). Immune-inflammatory functions of fibroblasts. Eur. Respir. J. 7, 2212–2222.

Kang, H.-B., Kim, Y.-E., Kwon, H.-J., Sok, D.-E., and Lee, Y. (2007). Enhancement of NF-kappaB expression and activity upon differentiation of human embryonic stem cell line SNUhES3. Stem Cells Dev. 16, 615–623.

Kanneganti, T.D., Lamkanfi, M., and Núñez, G. (2007). Intracellular NOD-like Receptors in Host Defense and Disease. Immunity 27, 549–559.

Kato, H., Takahasi, K., and Fujita, T. (2011). RIG-I-like receptors: Cytoplasmic sensors for non-self RNA. Immunol. Rev. 243, 91–98.

Kawai, T., and Akira, S. (2010). The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat. Immunol. 11, 373–384.

Kawai, T., and Akira, S. (2011). Toll-like Receptors and Their Crosstalk with Other Innate Receptors in Infection and Immunity. Immunity 34, 637–650.

Keller, G. (2005). Embryonic stem cell differentiation: Emergence of a new era in biology and medicine. Genes Dev. 19, 1129–1155.

Kim, H.S., Yun, J.W., Shin, T.H., Lee, S.H., Lee, B.C., Yu, K.R., Seo, Y., Lee, S., Kang, T.W., Choi, S.W., et al. (2015). Human umbilical cord blood mesenchymal stem cell-derived PGE2 and TGF-β1 alleviate atopic dermatitis by reducing mast cell degranulation. Stem Cells 33, 1254–1266.

Kim, Y.-E., Kang, H.-B., Park, J.-A., Nam, K.-H., Kwon, H.-J., and Lee, Y. (2008). Upregulation of NF-kappaB upon differentiation of mouse embryonic stem cells. BMB Rep. 41, 705–709.

Kimbrel, E.A., Kouris, N.A., Yavanian, G.J., Chu, J., Qin, Y., Chan, A., Singh, R.P., McCurdy, D., Gordon, L., Levinson, R.D., et al. (2014). Mesenchymal stem cell population derived from human pluripotent stem cells displays potent immunomodulatory and therapeutic properties. Stem Cells Dev. 23, 1611–1624.

Kündig, T.M., Bachmann, M.F., DiPaolo, C., Simard, J.J., Battegay, M., Lother, H., Gessner, a, Kühlcke, K., Ohashi, P.S., and Hengartner, H. (1995). Fibroblasts as efficient antigen-presenting cells in lymphoid organs. Science 268, 1343–1347.

Kurt-Jones, E.A., Sandor, F., Ortiz, Y., Bowen, G.N., Counter, S.L., Wang, T.C., and Finberg, R.W. (2004). Use of murine embryonic fibroblasts to define Toll-like receptor activation and specificity. J. Endotoxin Res. 10, 419–424.

Ledebur, H., and Parks, T. (1995). Transcriptional Regulation of the Intercellular Adhesion Molecule-1 Gene by Inflammatory Cytokines in Human Endothelial Cells ESSENTIAL ROLES OF A VARIANT. J. Biol. Chem. 270, 933–943.

Lee, J., Sayed, N., Hunter, A., Au, K.F., Wong, W.H., Mocarski, E.S., Pera, R.R., Yakubov, E., and Cooke, J.P. (2012). Activation of innate immunity is required for efficient nuclear reprogramming. Cell 151, 547–558.

Lee, S.-H., Hong, B., Sharabi, A., Huang, X.F., and Chen, S.-Y. (2009). Embryonic stem cells and mammary luminal progenitors directly sense and respond to microbial products. Stem Cells 27, 1604–1615.

Levenberg, S., Golub, J.S., Amit, M., Itskovitz-Eldor, J., and Langer, R. (2002). Endothelial cells derived from human embryonic stem cells. Proc. Natl. Acad. Sci. U. S. A. 99, 4391–4396.

Levy, O. (2007). Innate immunity of the newborn: basic mechanisms and clinical correlates. Nat. Rev. Immunol. 7, 379–390.

Li, L., Baroja, M.L., Majumdar, A., Chadwick, K., Rouleau, A., Gallacher, L., Ferber, I., Lebkowski, J., Martin, T., Madrenas, J., et al. (2004). Human embryonic stem cells possess immune-privileged properties. Stem Cells 22, 448–456.

Li, Y., Lu, J., Han, Y., Fan, X., and Ding, S.-W. (2013). RNA interference functions as an antiviral immunity mechanism in mammals. Science 342, 231–234.

Libermann, T.A., and Baltimore, D. (1990). Activation of interleukin-6 gene expression through the NF-kappa B transcription factor. Mol. Cell. Biol. 10, 2327–2334.

Van Linthout, S., Miteva, K., and Tschöpe, C. (2014). Crosstalk between fibroblasts and inflammatory cells. Cardiovasc. Res. 102, 258–269.

Liu, J.W., Dunoyer-Geindre, S., Serre-Beinier, V., Mai, G., Lambert, J.F., Fish, R.J., Pernod, G., Buehler, L., Bounameaux, H., and Kruithof, E.K.O. (2007). Characterization of endothelial-like cells derived from human mesenchymal stem cells. J. Thromb. Haemost. 5, 826–834.

Lorenz, K., Sicker, M., Schmelzer, E., Rupf, T., Salvetter, J., Schulz-Siegmund, M., and Bader, A. (2008). Multilineage differentiation potential of human dermal skin-derived fibroblasts. Exp. Dermatol. 17, 925–932.

Lüningschrör, P., Stöcker, B., Kaltschmidt, B., and Kaltschmidt, C. (2012). miR-290 cluster modulates pluripotency by repressing canonical NF-κB signaling. Stem Cells 30, 655–664.

Ma, M., Ding, S., Lundqvist, A., San, H., Fang, F., Konoplyannikov, M., Berry, C., Beltran, L.E., Chen, G., Kovacic, J.C., et al. (2010). Major histocompatibility complex-I expression on embryonic stem cell-derived vascular progenitor cells is critical for syngeneic transplant survival. Stem Cells 28, 1465–1475.

Ma, S., Xie, N., Li, W., Yuan, B., Shi, Y., and Wang, Y. (2014). Immunobiology of mesenchymal stem cells. Cell Death Differ. 21, 216–225.

Maggini, J., Mirkin, G., Bognanni, I., Holmberg, J., Piazzón, I.M., Nepomnaschy, I., Costa, H., Cañones, C., Raiden, S., Vermeulen, M., et al. (2010). Mouse bone marrow-derived mesenchymal stromal cells turn activated macrophages into a regulatory-like profile. PLoS One 5, e9252.

Magliocca, J.F., Held, I.K. a, and Odorico, J.S. (2006). Undifferentiated murine embryonic stem cells cannot induce portal tolerance but may possess immune privilege secondary to reduced major histocompatibility complex antigen expression. Stem Cells Dev. 15, 707–717.

Mai, J., Virtue, A., Shen, J., Wang, H., and Yang, X.-F. (2013). An evolving new paradigm: endothelial cells – conditional innate immune cells. J. Hematol. Oncol. 6, 61.

Maillard, P. V, Ciaudo, C., Marchais, A., Li, Y., Jay, F., Ding, S.W., and Voinnet, O. (2013). Antiviral RNA interference in mammalian cells. Science 342, 235–238.

Majore, I., Moretti, P., Hass, R., and Kasper, C. (2009). Identification of subpopulations in mesenchymal stem cell-like cultures from human umbilical cord. Cell Commun. Signal. 7, 6.

Matsumoto, M., and Seya, T. (2008). TLR3: Interferon induction by double-stranded RNA including poly(I:C). Adv. Drug Deliv. Rev. 60, 805–812.

McCloskey, K.E., Stice, S.L., and Nerem, R.M. In Vitro Derivation and Expansion of Endothelial Cells From Embryonic Stem Cells. In Embryonic Stem Cell Protocols, (New Jersey: Humana Press), pp. 287–302.

Medzhitov, R. (2008). Origin and physiological roles of inflammation. Nature 454, 428–435.

Medzhitov, R. (2010). Inflammation 2010: New Adventures of an Old Flame. Cell 140, 771–776.

Nathan, C. (2002). Points of control in inflammation. Nature 420, 846–852.

Di Nicola, M., Carlo-Stella, C., Magni, M., Milanesi, M., Longoni, P.D., Matteucci, P., Grisanti, S., and Gianni, A.M. (2002). Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. Blood 99, 3838–3843.

Niwa, H., Burdon, T., Chambers, I., and Smith, A. (1998). Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3. Genes Dev 12, 2048–2060.

Olivier, E.N., Rybicki, A.C., and Bouhassira, E.E. (2006). Differentiation of human embryonic stem cells into bipotent mesenchymal stem cells. Stem Cells 24, 1914–1922.

Owen, M. (1988). Marrow stromal stem cells. J. Cell Sci. 10, 63–76.

Pan, Z.K., Fisher, C., Li, J.D., Jiang, Y., Huang, S., and Chen, L.Y. (2011). Bacterial LPS up-regulated TLR3 expression is critical for antiviral response in human monocytes: Evidence for negative regulation by CYLD. Int. Immunol. 23, 357–364.

Pare, J.M., and Sullivan, C.S. (2014). Distinct antiviral responses in pluripotent versus differentiated cells. PLoS Pathog. 10, e1003865.

Pfaffl, M.W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29, 45e–45.

Pinney, D.F., and Emerson, C.P. (1989). 10T1/2 cells: An in vitro model for molecular genetic analysis of mesodermal determination and differentiation. Environ. Health Perspect. 80, 221–227.

Prockop, D.J., and Youn Oh, J. (2012). Mesenchymal Stem/Stromal Cells (MSCs): Role as Guardians of Inflammation. Mol. Ther. 20, 14–20.

Qian, L., and Saltzman, W.M. (2004). Improving the expansion and neuronal differentiation of mesenchymal stem cells through culture surface modification. Biomaterials 25, 1331–1337.

Rajan, S., Ye, J., Bai, S., Huang, F., and Guo, Y.-L. (2008). NF-kappaB, but not p38 MAP kinase, is required for TNF-alpha-induced expression of cell adhesion molecules in endothelial cells. J. Cell. Biochem. 105, 477–486.

Randall, R.E., and Goodbourn, S. (2008). Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures. J. Gen. Virol. 89, 1–47.

Rath, M., Müller, I., Kropf, P., Closs, E.I., and Munder, M. (2014). Metabolism via arginase or nitric oxide synthase: Two competing arginine pathways in macrophages. Front. Immunol. 5.

Reger, R.L., Tucker, A.H., and Wolfe, M.R. (2008). Differentiation and characterization of human MSCs. Methods Mol. Biol. 449, 93–107.

Ren, G., Zhang, L., Zhao, X., Xu, G., Zhang, Y., Roberts, A.I., Zhao, R.C., and Shi, Y. (2008). Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide. Cell Stem Cell 2, 141–150.

Reznikoff, C.A., Brankow, D.W., and Heidelberger, C. (1973). Establishment and characterization of a cloned line of C3H mouse embryo cells sensitive to postconfluence inhibition of division. Cancer Res. 33, 3231–3238.

Ryan, G.B., and Majno, G. (1977). Acute inflammation. A review. Am. J. Pathol. 86, 185–274.

Sacre, S.M., Lundberg, A.M.C., Andreakos, E., Taylor, C., Feldmann, M., and Foxwell, B.M. (2007). Selective Use of TRAM in Lipopolysaccharide (LPS) and Lipoteichoic Acid (LTA) Induced NF- B Activation and Cytokine Production in Primary Human Cells: TRAM Is an Adaptor for LPS and LTA Signaling. J. Immunol. 178, 2148–2154.

Samuel, C.E. (2001). Antiviral actions of interferons. Clin. Microbiol. Rev. 14, 778–809.

Sato, K., Ozaki, K., Oh, I., Meguro, A., Hatanaka, K., Nagai, T., Muroi, K., and Ozawa, K. (2007). Nitric oxide plays a critical role in suppression of T-cell proliferation by mesenchymal stem cells. Blood 109, 228–234.

Schlee, M. (2013). Master sensors of pathogenic RNA - RIG-I like receptors. Immunobiology 218, 1322–1335.

Schroder, K., Hertzog, P.J., Ravasi, T., and Hume, D.A. (2004). Interferon-gamma: an overview of signals, mechanisms and functions. J. Leukoc. Biol. 75, 163–189.

Sedger, L.M., and McDermott, M.F. (2014). TNF and TNF-receptors: From mediators of cell death and inflammation to therapeutic giants - past, present and future. Cytokine Growth Factor Rev. 25, 453–472.

Sen, G.C. (2001). Viruses and Interferons. Annu. Rev. Microbiol. 55, 255–281.

Shi, Y., Hu, G., Su, J., Li, W., Chen, Q., Shou, P., Xu, C., Chen, X., Huang, Y., Zhu, Z., et al. (2010). Mesenchymal stem cells: a new strategy for immunosuppression and tissue repair. Cell Res. 20, 510–518.

Sidney, L.E., Kirkham, G.R., and Buttery, L.D. (2014). Comparison of osteogenic differentiation of embryonic stem cells and primary osteoblasts revealed by responses to IL-1β, TNF-α, and IFN-γ. Stem Cells Dev. 23, 605–617.

Sinha, S., Hoofnagle, M.H., Kingston, P. a, Mccanna, M.E., Owens, G.K., and Mary, E. (2004). Transforming growth factor- beta 1 signaling contributes to development of smooth muscle cells from embryonic stem cells. Am. J. Physiol. Cell Physiol. 22908, 1560–1568.

Smith, R.S., Smith, T.J., Blieden, T.M., and Phipps, R.P. (1997). Fibroblasts as sentinel cells. Synthesis of chemokines and regulation of inflammation. Am. J. Pathol. 151, 317–322.

Soldan, S.S., Hollidge, B.S., Wagner, V., Weber, F., and González-Scarano, F. (2010). La Crosse virus (LACV) Gc fusion peptide mutants have impaired growth and fusion phenotypes, but remain neurotoxic. Virology 404, 139–147.

Swartzendruber, D.E., and Lehman, J.M. (1975). Neoplastic differentiation: interaction of simian virus 40 and polyoma virus with murine teratocarcinoma cells in vitro. J Cell Physiol 85, 179–187.

Swartzendruber, D.E., Friedrich, T.D., and Lehman, J.M. (1977). Resistance of teratocarcinoma stem cells to infection with simian virus 40: early events. J. Cell. Physiol. 93, 25–30.

Swijnenburg, R.-J., Tanaka, M., Vogel, H., Baker, J., Kofidis, T., Gunawan, F., Lebl, D.R., Caffarelli, A.D., de Bruin, J.L., Fedoseyeva, E. V., et al. (2005). Embryonic stem cell immunogenicity increases upon differentiation after transplantation into ischemic myocardium. Circulation 112, I166-72.

Tabar, V., and Studer, L. (2014). Pluripotent stem cells in regenerative medicine: challenges and recent progress. Nat. Rev. Genet. 15, 82–92.

Takano, T., Li, Y.-J., Kukita, A., Yamaza, T., Ayukawa, Y., Moriyama, K., Uehara, N., Nomiyama, H., Koyano, K., and Kukita, T. (2014). Mesenchymal stem cells markedly suppress inflammatory bone destruction in rats with adjuvant-induced arthritis. Lab. Investig. 94, 286–296.

Tan, Y., Ooi, S., and Wang, L. (2014). Immunogenicity and tumorigenicity of pluripotent stem cells and their derivatives: genetic and epigenetic perspectives. Curr. Stem Cell Res. Ther. 9, 63–72.

Taylor, T., Kim, Y.-J., Ou, X., Derbigny, W., and Broxmeyer, H.E. (2010). Toll-like receptor 2 mediates proliferation, survival, NF-kappaB translocation, and cytokine mRNA expression in LIF-maintained mouse embryonic stem cells. Stem Cells Dev. 19, 1333–1341.

Thomson, J.A., Itskovitz-Eldor, J., Shapiro, S., Waknitz, M., Swiergiel, J., Marshall, V., and Jones, J. (1998). Embryonic Stem Cell Lines Derived from Human Blastocysts. Science (80-.). 282, 1145–1147.

Torres, J., and Watt, F.M. (2008). Nanog maintains pluripotency of mouse embryonic stem cells by inhibiting NFkappaB and cooperating with Stat3. Nat. Cell Biol. 10, 194–201.

U.S. National Institutes of Health (2016). ClinicalTrials.gov.

Ulrich, C. (2012). Mesenchymal Stromal Cells and Fibroblasts. J. Tissue Sci. Eng. 3, 1–6.

de Veer, M.J., Holko, M., Frevel, M., Walker, E., Der, S., Paranjape, J.M., Silverman, R.H., and Williams, B.R. (2001). Functional classification of interferon-stimulated genes identified using microarrays. J Leukoc Biol 69, 912–920.

Virchow, R. (1858). Die Cellularpathologie in ihrer Begründung auf physiologische and pathologische Gewebelehre. Verlag von August Hirschfeld, Berlin. 1858.

Wada, N., Bartold, P.M., and Gronthos, S. (2011). Human foreskin fibroblasts exert immunomodulatory properties by a different mechanism to bone marrow stromal/stem cells. Stem Cells Dev. 20, 647–659.

Wada, N., Gronthos, S., and Bartold, P.M. (2013). Immunomodulatory effects of stem cells. Periodontol. 2000 63, 198–216.

Wagner, W., Wein, F., Seckinger, A., Frankhauser, M., Wirkner, U., Krause, U., Blake, J., Schwager, C., Eckstein, V., Ansorge, W., et al. (2005). Comparative characteristics of mesenchymal stem cells from human bone marrow, adipose tissue, and umbilical cord blood. Exp. Hematol. 33, 1402–1416.

Wakitani, S., Saito, T., and Caplan, A.I. (1995). Myogenic cells derived from rat bone marrow mesenchymal stem cells exposed to 5-azacytidine. Muscle Nerve 18, 1417–1426.

Wang, J., Rao, S., Chu, J., Shen, X., Levasseur, D.N., Theunissen, T.W., and Orkin, S.H. (2006). A protein interaction network for pluripotency of embryonic stem cells. Nature 444, 364–368.

Wang, R., Wang, J., Paul, A.M., Acharya, D., Bai, F., Huang, F., and Guo, Y.-L. (2013). Mouse embryonic stem cells are deficient in type I interferon expression in response to viral infections and double-stranded RNA. J. Biol. Chem. 288, 15926–15936.

Wang, R., Teng, C., Spangler, J., Wang, J., Huang, F., and Guo, Y.-L. (2014a). Mouse Embryonic Stem Cells Have Underdeveloped Antiviral Mechanisms That Can Be Exploited for the Development of mRNA-Mediated Gene Expression Strategy. Stem Cells Dev. 23, 594–604.

Wang, R., Wang, J., Acharya, D., Paul, A.M., Bai, F., Huang, F., and Guo, Y.-L. (2014b). Antiviral responses in mouse embryonic stem cells: differential development of cellular mechanisms in type I interferon production and response. J. Biol. Chem. 289, 25186–25198.

Wash, R., Calabressi, S., Franz, S., Griffiths, S.J., Goulding, D., Tan, E.-P., Wise, H., Digard, P., Haas, J., Efstathiou, S., et al. (2012). Permissive and restricted virus infection of murine embryonic stem cells. J. Gen. Virol. 93, 2118–2130.

Wobus, A.M., and Boheler, K.R. (2005). Embryonic Stem Cells : Prospects for Developmental Biology and Cell Therapy. Physiol. Rev. 85, 635–678.

Wuu, Y., Pampfer, S., Vanderheyden, I., Lee, K., and Hertogh, R. De (1998). Impact of tumor necrosis factor alpha on mouse embryonic stem cells. Biol. Reprod. 1424, 1416–1424.

Yamashita, J., Itoh, H., Hirashima, M., Ogawa, M., Nishikawa, S., Yurugi, T., Naito, M., Nakao, K., and Nishikawa, S. (2000). Flk1-positive cells derived from embryonic stem cells serve as vascular progenitors. Nature 408, 92–96.

Yoneyama, M., and Fujita, T. (2007). Function of RIG-I-like receptors in antiviral innate immunity. J. Biol. Chem. 282, 15315–15318.

Yoneyama, M., Kikuchi, M., Natsukawa, T., Shinobu, N., Imaizumi, T., Miyagishi, M., Taira, K., Akira, S., and Fujita, T. (2004). The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. Nat. Immunol. 5, 730–737.

Yu, J., Rossi, R., Hale, C., Goulding, D., and Dougan, G. (2009). Interaction of enteric bacterial pathogens with murine embryonic stem cells. Infect. Immun. 77, 585–597.

Yu, L., Wang, L., and Chen, S. (2010). Endogenous toll-like receptor ligands and their biological significance. J. Cell. Mol. Med. 14, 2592–2603.

Zampetaki, A., Xiao, Q., Zeng, L., Hu, Y., and Xu, Q. (2006). TLR4 expression in mouse embryonic stem cells and in stem cell-derived vascular cells is regulated by epigenetic modifications. Biochem. Biophys. Res. Commun. 347, 89–99.

Zampetaki, A., Zeng, L., Xiao, Q., Margariti, A., Hu, Y., and Xu, Q. (2007). Lacking cytokine production in ES cells and ES-cell-derived vascular cells stimulated by TNF-alpha is rescued by HDAC inhibitor trichostatin A. Am. J. Physiol. Cell Physiol. 293, C1226-38.

Zhang, M., Mal, N., Kiedrowski, M., Chacko, M., Askari, A.T., Popovic, Z.B., Koc, O.N., and Penn, M.S. (2007). SDF-1 expression by mesenchymal stem cells results in trophic support of cardiac myocytes after myocardial infarction. FASEB J. 21, 3197–3207.